The Potential Role of *Wolbachia* as a Defensive Mutualist in the *Onchocerca ochengi* Nodule

---

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

Rowena Dawn Ellen Hansen

October 2011

ABSTRACT

The Potential Role of *Wolbachia* as a Defensive Mutualist in the *Onchocerca ochengi* Nodule

Rowena Dawn Ellen Hansen

Onchocerciasis (River Blindness) is a continuing major cause of human blindness, skin disease and socioeconomic impediment in sub-Saharan Africa and Latin America. An estimated 37 million people are infected and a further 89 million are at risk. Human onchocerciasis is caused by the filarial nematode *Onchocerca volvulus*, and is spread by the blackfly vector *Simulium* spp, found in riverine areas, encompassing land which is agriculturally valuable. Ivermectin is currently distributed in these areas to control the microfilarial stage of the parasite, but adult worm viability is unaffected. Thus, due to the long lifespan of the worms (>10 years), treatment regimens must be continued for many years. Furthermore, there are signs that ivermectin resistance is emerging.

The bovine filarial parasite *Onchocerca ochengi* represents the closest phylogenetic relative of *O. volvulus*. In both species, female worms reside in nodules, and continue to reproduce for many years whilst surrounded by a milieu of host inflammatory cells and antibodies. In addition, both species harbour endosymbiotic bacteria (*Wolbachia*) which are essential for worm survival. Although a metabolic role for these bacteria has been hypothesised, the symbiotic mechanisms between filariae and *Wolbachia* are not fully understood. In the filarial nodule, neutrophils are attracted to *Wolbachia*-rich worms, but if the bacteria are depleted via antibiotic chemotherapy, the neutrophils are replaced by eosinophils that degranulate on the worm cuticle. However, the specific contribution of eosinophils towards filarial death is unresolved.

This study sought to define the role of eosinophils in either the active killing or post-killing clearance of *Wolbachia*-depleted filariae. In an *in-vivo* longitudinal study of *O. ochengi*-infected cattle receiving adulticidal regimens of oxytetracycline or melarsomine, histopathological analysis of nodules revealed a switch from neutrophils to eosinophils in oxytetracycline-treated animals, but not following melarsomine treatment, and quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) demonstrated that viable *Wolbachia* were depleted only by oxytetracycline chemotherapy. Eosinophil degranulation increased significantly only in the oxytetracycline group; whereas nodular gene expression of bovine neutrophilic chemokines was lowest in this group. Moreover, intense eosinophil degranulation was initially associated with worm vitality, not degeneration, while transmission electron microscopy (TEM) revealed subsequent cuticular damage in *Wolbachia*-depleted worms. Taken together, these data offer strong support for the hypothesis that *Wolbachia* confers longevity on *O. ochengi* through a defensive mutualism, in which neutrophils attracted to *Wolbachia* divert a potentially lethal effector response by eosinophils.

ACKNOWLEDGEMENTS

I am sincerely grateful to my supervisors for their support, guidance and advice throughout this work. I thank Dr Ben Makepeace for his attentive supervision and constructive critiquing both in the laboratory and in the office; and Professor A. J Trees for investing so much time and knowledge overseeing each aspect of the project. I would also like to thank Professor Diana Williams for her support, and the people of the Veterinary Parasitology department for their friendliness, words of encouragement and assistance in the laboratory. I am particularly grateful to Catherine Hartley for lending her laboratory experience, and for her friendship.

I am grateful to Dr Odile Bain for her authoritative tuition on filarial morphology, and to Dr Coralie Martin for her advice on cell migration assays. I am equally grateful to Professor Judith Allen for her constructive advice, and to Dr Simon Babayan for welcoming me at the Allen Laboratory. I am indebted to Dr Udo Hetzel for his tuition on optical and electron microscopic techniques, and for sharing his office during the long hours of granulocyte quantification. I would also like to thank Marion Pope and the staff of Veterinary Histology for their technical support with electron microscopy, and preparation of histological sections, respectively.

Much of this work could not have been achieved without the help of our African partners. I am very thankful to Dr Vincent Tanya, who was integral for solving logistical problems in Cameroon. I am grateful to Germanus S. Bah, David Ekale and Henrietta Ngangyung, the team based at the Institut de Recherche Agricole pour le Développment (IRAD), Regional Centre at Wakwa, Ngaoundéré (North Cameroon), for their vital technical assistance with the *in-vivo* experiment. Additionally, I would like to thank the herdsmen of IRAD for keeping the herd safe and healthy and for their invaluable assistance in cattle handling. I equally thank Valentine for keeping us safe on the Cameroonian roads.

The preliminary work on the cell migration assays could not have occurred without the kind help from the meat inspectors and abattoir workers at Taylor's, Bamber Bridge, Leyland, who collected fresh cattle blood. For the definitive cell migration assays, I am grateful to Nigel Jones and his team at Ness Heath Farm (Neston, Wirral) for their assistance with handling the donor bull.

This project also received support from Frans Van Gool (Merial), who coordinated the donation of Cymelarsan; and Dr Casiraghi (University of Milan, Italy) who donated the rabbit polyclonal antibody which was used for immunohistochemistry. I am grateful to Theo Kanellos (Pfizer Animal Health), for his input in to the preparation of a poster on this work. I would also like to express my gratitude to the BBSRC for the receipt of a doctoral training award.

I owe very special thanks to my family for their continued love and support throughout. I thank my husband for his companionship and words of encouragement, and my daughter for her smiles. I am indebted to my parents for their practical help during my thesis writing, and to James for his technical support and advice on the final document.

As a veterinarian, I would like to acknowledge the animals used in our experimental procedures. Without them, this work would not have existed.

This work was supported by Pfizer Animal Health and the EC (contract no. INCO-CT-2006-032321)

DEDICATION

To my husband, Nicholas, who made this work endurable;
to my parents, who made it possible;
and to Lilian, who makes it all worthwhile.

LIST OF ABBREVIATIONS

A	Aa	*Aedes albopictus*
	APOC	African Programme for Onchocerciasis Control
	ATP	adenosine triphosphate
	aWSP	anti-*Wolbachia*-surface protein antibodies
B	BD	Becton Dickinson
	BSA	bovine serum albumin
C	C3a	complement 3a
	C5a	complement 5a
	CC	cysteine-cystiene chemokine
	CD	cluster of differentiation
	cDNA	complementary DNA
	CDTI	community-directed treatment with ivermectin
	CGP-20376	3-[(2-tert-butyl-5-methoxy-1,3-benzothiazol-6-yl) carbamothioylsulfanyl] propanoic acid
	CI	cytoplasmic incompatibility
	CK	chemokinesis
	cm	centimetre
	COM	combined oxytetracycline chemotherapy
	CON	control
	CT	chemotaxis
	CXC	cystiene-X-cysteine chenokine
	CXCL	Chemokine (C-X-C motif) ligand
	CXCR	Chemokine (C-X-C motif) receptor
D	DAB	diaminobenzidine
	DE	degranulating eosinophils
	DEC	diethylcarbamazine
	desArg	without arginine
	DMEM	Dulbecco's Modified Eagle Medium
	DNA	deoxyribonucleic acid
	dNTP	deoxyribonucleotide phosphate
	DOX	doxycycline
	DPX	distyrene-tricresyl phosphate-xylene
E	ECP	eosinophil cationic protein
	EDN	eosinophil-derived neurotoxin
	EDTA	Ethylenediaminetetraacetic acid
	ELISA	enzyme-linked immunosorbent assay
	ELR	glutamic acid-leucine-arginine motif
	EM	electron microscopy
	ENA-78	epithelial neutrophil activating peptide-78
	EPO	eosinophil peroxidase
	E/S	excretory/secretory
	E-selectin	endothelial selectin
	ES-62	excretory secretory product 62
	et al.	and others
F	Fc	crystallisable fragment
	fMLP	N-formylmethionyl-leucyl-phenylalanine
	FOXP3	forkhead box P3
G	g	gram

	g	gravitational force
	GLM	general linear model
	GluCl	glutamate-gated chloride channel
	GM-CSF	granulocyte-macrophage colony-stimulating factor
	GRO	growth-regulated oncogene
	GroEL	Heat shock 60kDa protein
	GST	glutathione S-transferase
I	ICAM	inter-cellular adhesion molecule
	IFN-γ	interferon-gamma
	IHC	immunohistochemistry
	Ig	immunoglobulin
	IL	interleukin
	IRAD	Institut de Recherche Agricole pour le Développment
	IV	intravenous
	IVM	ivermectin
K	KC	Keratinocyte-derived cytokine
	kDa	kiloDalton
	kg	kilogram
	kHz	kilohertz
	km	kilometre
L	L	litre
	L1	first-stage larva (microfilaria)
	L3	infective (third stage) larva
	L4	fourth-stage larva
	LPS	lipopolysaccharide
	LT	leukotriene
M	M	molar
	MBP	major basic protein
	MEL	melarsomine
	mff	microfilaria
	mg	milligram
	MHC	major histocompatibility complex
	MIC	minimum inhibitory concentration
	MIF	macrophage migration inhibitory factor
	ml	millilitre
	mm	millimetre
	mM	miliMolar
	MPO	myeloperoxidase
	mRNA	messenger ribonucleic acid
	MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
	MyD88	Myeloid differentiation primary response gene (88)
N	nm	nanometres
	NTC	no treatment controls
O	*Oo*GST	*Onchocerca ochengi* glutathione S-transferase
	*Ov*GST	*Onchocerca volvulus* glutathione S-transferase
	OXY	oxytetracycline
P	*p*	probability
	PAF	platelet activating factor
	PAL	peptidoglycan-associated lipoprotein
	PAMP	pathogen-associated molecular pattern

	PBMC	peripheral blood mononuclear cells
	PBS	phosphate buffered saline
	PCR	polymerase chain reaction
	PDG	peptidoglycan
	PECAM	platelet endothelial cell adhesion molecule
	PMN	polymorphonuclear leukocyte
	P-selectin	platelet-selectin
Q	qRT-PCR	quantitative reverse-transcriptase polymerase chain reaction
R	RANTES	regulated upon activation, normal T cell expressed and secreted)
	*rb*IL-8	recombinant bovine IL-8
	*rm*eotaxin	recombinant murine eotaxin
	RNA	ribonucleic acid
	rpm	revolutions per minute
	RPMI	Roswell Park Memorial Institute medium
	rRNA	ribosomal ribonucleic acid
	RT	reverse transcriptase
	rWSP	recombinant *Wolbachia* surface protein
S	SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
	SID	once daily
	SIR	short intensive regimen
	spp.	species
	ssRNA	single stranded ribonucleic acid
T	TEM	transmission electron microscope
	TGF	transforming growth factor
	TGS	Tris-glycine-SDS
	Th	T helper cell
	TLR	toll-like receptor
	T_m	melting temperature
	TNF-α	tumour necrosis factor alpha
	tp	time point
	TPX	thioredoxin peroxidase
	T-reg	T –regulatory cell
	tRNA	transfer RNA
U	U	units
	UK	United Kingdom
	UMF-078	methyl (±)-[5-(α-amino-4-fluorobenzyl) benzimidazol-2-yl] carbamate
V	VEGF	vascular endothelial growth factor
W	WHO	World Health organisation
	*w*Mel	*Wolbachia* strain *Melanogaster*
	wpt	weeks post-treatment
	WSP	*Wolbachia* surface protein
	w/v	weight per volume
Z	ZAS	zymosan-activated serum
	°C	degrees Celsius
	1°	primary
	2°	secondary
	3°	tertiary
	µg	microgram

µl	microlitre
µm	micrometre
♀	female
♂	male

CONTENTS

1. Review of the literature

1.1. Filarial parasites of human and veterinary importance

Filarial nematodes are a major cause of global morbidity in humans, and the most important filarial diseases are onchocerciasis, lymphatic filariasis and loiasis. Onchocerciasis (river blindness), caused by *Onchocerca volvulus* leads to debilitating eye and skin pathology, and an estimated 37 million people are currently infected (Basanez et al., 2006). Endemic foci of *O. volvulus* exist mainly within areas of West and Central Africa, although areas of South and Central America are also affected. *O. volvulus* adults live and breed in subcutaneous nodules, located mainly over bony prominences. However, it is the microfilariae that cause the significant ocular (Donnelly et al., 1985) and dermal (Murdoch et al., 1993) pathological manifestations and associated socioeconomic impact (Evans, 1995), which has culminated in an estimated half a million disability-adjusted life-years (Mathers et al., 2007). Lymphatic filariasis (or elephantiasis), caused by *Wuchereria bancrofti* and *Brugia* spp., is prevalent throughout the tropics, and causes pathology as a result of damage to the lymphatic system. Pathological sequelae include swelling of the limbs (lymphoedema) and scrotum (hydrocoele), culminating in an estimated 5.5 million disability-adjusted life-years (Mathers et al., 2007). Loiasis, caused by *Loa loa*, affects millions of people in Central Africa. Ocular pathology caused by worm migratory activity under the conjunctiva manifests as pruritus, photophobia and sometimes ocular oedema; and Calabar swellings, which are usually apparent on the limbs, are associated with localised prutitus and restricted joint function (Boussinesq, 2006).

The most prominent filaria of clinical veterinary importance is the heartworm *Dirofilaria immitis*, which is prevalent worldwide in warm climates, and also seasonally in temperate climates (Genchi et al., 2005). *D. immitis* resides within the pulmonary arteries and the right ventricle of infected dogs and cats, causing circulatory insult, which leads to congestive heart failure in heavily-infected animals. *D. immitis* is also zoonotic (although the incidence of zoonosis is very low), and as worms are usually encompassed within granulomatous 'coin lesions' within the human lung, they are usually only noticed during routine lung radiography, or at post-mortem examination (Gutierrez, 1984).

Many other filariae affect animals but are associated with little or no pathology, and whilst they are not considered to be veterinary pathogens, their indirect value as a tool for use within comparative research models has enabled the advancement of knowledge in important human filarial diseases. Examples are the *Onchocerca ochengi* model of cattle, which is the closest analogue of *Onchocerca volvulus* (Bain, 1981); and *Litomosoides sigmodontis*, which is a natural parasite of the cotton rat, but is utilised in mice as a comparative model for onchocerciasis (Allen et al., 2008). A common feature of the life cycle of filarial parasites is their host-to-host transmission involving arthropod vectors. Lymphatic filariasis is transmitted by anopheline and culicine mosquitoes, whilst *O. volvulus* and *O. ochengi* share the same vector, the blackfly (*Simulium* spp.); and various species of tabanid flies (*Chrysops* spp.) transmit *L. loa*. During the lifecycle of filarial worms, microfilariae are ingested by their arthropod vector during a blood meal from an infected individual, then develop and moult twice within predilection sites specific to the filarial type. Infective larvae (L3) are passed onto the skin of the next definitive host via the proboscis during subsequent blood meals, where some succeed in entering

the wound caused by the vector. The L3 continue to develop and moult within the new vertebrate host, and mature worms start the process again once they are located in their specific niche within the definitive host. The lifecycles of *O. volvulus* and *O. ochengi* and their commonalities are illustrated in Figure 1.1.1

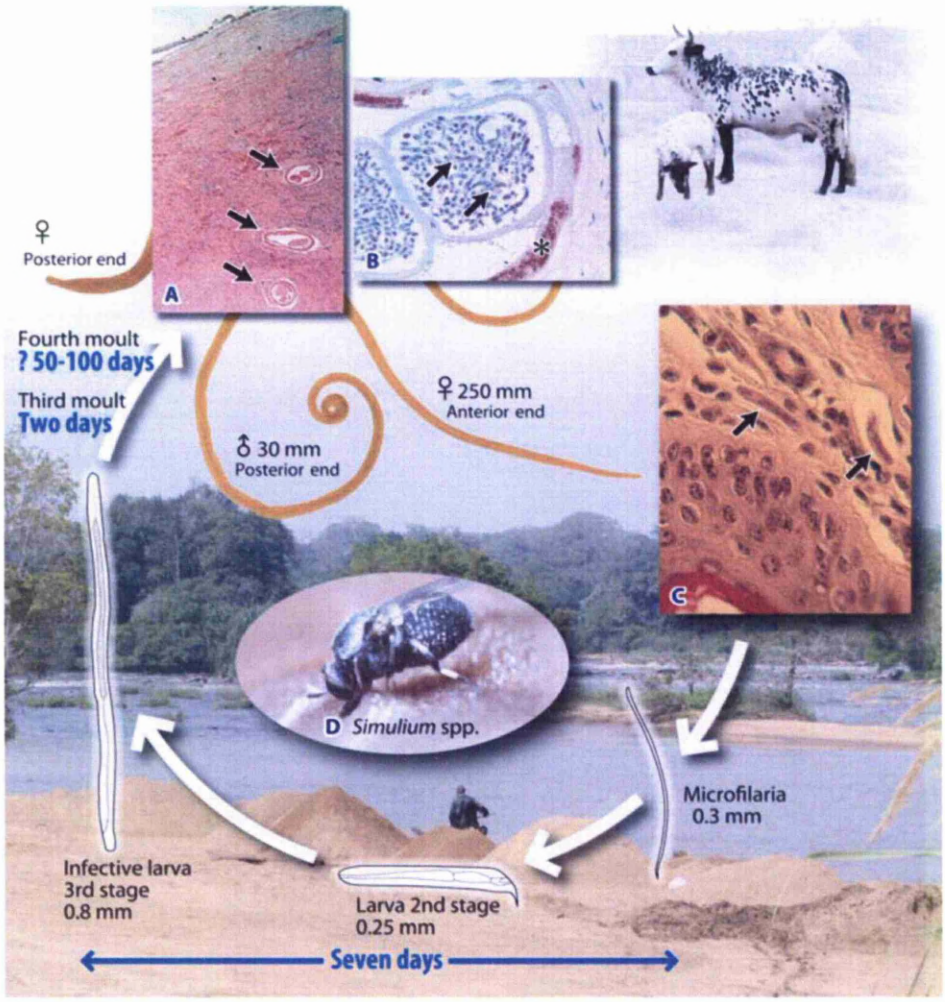


Figure 1.1.1. Life cycle of *O. volvulus* and *O. ochengi*. Adult female worms reside in subcutaneous (*O. volvulus*) or intradermal (*O. ochengi*) nodules, whilst producing millions of microfilariae (L1). Microfilariae migrate to the skin where they are ingested by blackfly vectors taking a blood meal from the vertebrate host. Microfilariae develop and moult to L2, then L3 over 7 days. Infective L3 are deposited on the skin during subsequent blood meals and enter via the bite wound. A and B: Transverse sections of female worms in onchocercoma. In (B), note the presence of *Wolbachia* (*) in the lateral hypodermal chords, and microfilariae within the paired uteri (arrows). C: presence of microfilariae (arrows) in the dermis of an infected individual. D: the *Simulium* (blackfly) vector. Reproduced from J. E Allen et al (2008) Of Mice, Cattle, and Humans: The Immunology and Treatment of River Blindness. PLoS Neglected Tropical Diseases **2** (4) e217.

1.2. The evolution of onchocerciasis control

Early onchocerciasis control consisted of an anti-vectorial strategy (aerial spraying of organophosphates in the early 1970s), and this was soon supplemented, then superseded by a reliance on anthelminthic drugs [mass donation and distribution of ivermectin from 1987 (Merck and Co., Inc)], which is now the only intervention used for most onchocerciasis foci in sub-Saharan Africa (Remme, 2004). The work of the World Health Organization (WHO), which instigated the Onchocerciasis Control Programme (from 1974 - 2002) and then the African Programme for Onchocerciasis Control [(APOC) 1995 - ongoing], has been very successful in reducing morbidity, since annual Community–Directed Treatment with Ivermectin (CDTI) suppresses microfilarial densities in the skin below the level at which they cause disease (Remme, 2004; Tielsch and Beeche, 2004).

Although APOC has drastically improved the lives of many thousands of people in sub-Saharan Africa, an estimated 37 million people remain infected with *O. volvulus* (Basanez et al., 2006). Ivermectin is not curative; it has only a limited cumulative effect on the adult worm and even after treatment for 6 years, most worms are still alive and able to reproduce (Klager et al., 1996), and partial repopulation of the skin by microfilariae has been reported (WHO 1995). Ivermectin imparts only limited effects on embryogenesis: although it kills mature microfilariae effectively, it has little effect on earlier stages, allowing continual replenishment of the microfilarial load (Hoerauf et al., 2001). In addition, the use of ivermectin in *Loa loa* endemic areas is restricted because of the risk of severe side effects (including fatal encephalopathy) in heavily co-infected patients (Gardon et al., 1997). Moreover, recent studies have reported that following ivermectin treatment within Ghanaian communities, some individuals show a minimal interruption of embryogenesis and

active intra-uterine microfilarial production, followed by a rapid repopulation of the skin by microfilariae, providing evidence for emerging ivermectin resistance (Osei-Atweneboana et al., 2011).

Other antifilarial drugs that have been implemented in the treatment of human onchocerciasis include diethylcarbamazine (DEC) and suramin. Diethylcarbamazine is thought to unmask microfilarial antigens, rendering them exposed to immunological attack (Piessens and Beldekas, 1979). DEC exhibits macrofilaricidal properties in *Wuchereria bancrofti* scrotal worm nests, demonstrated by a decrease in the 'filarial dance sign' upon ultrasonographic examination (Noroes et al., 1997). However, this study also reported that DEC was only found to be effective against a proportion of worms studied (Noroes et al., 1997). Furthermore, the drug was not found to have any long-term macro- or microfilaricidal effects on *O. volvulus* (Albiez et al., 1988). DEC causes a severe multifaceted syndrome (including hypotension, pruritus, fever, adenitis, arthralgia, and tachycardia; collectively called the 'Mazzotti reaction') in some patients, and the intensity of this may be correlated with the microfilarial load (Francis et al., 1985). Suramin is effective at clearing microfilariae and resolving nodules in onchocerciasis patients treated with the WHO recommended regimen (Chijioke et al., 1998), and in *O. ochengi* it has been shown to attain concentrations in nodule tissue that exceed those in skin (Cross et al., 1997). However, suramin is a highly toxic agent with a narrow therapeutic index, and causes serious adverse reactions including fatal collapse in some patients; although overdosing has been blamed for a proportion of these deaths (Awadzi, 2003). Because of the high risk to human health and the unacceptable clinical side effects of these drugs, ivermectin has superseded the use of DEC and suramin within affected communities today. However, the rediscovery of bacterial endosymbionts *Wolbachia*

within filarial species, and the recent realisation of the significance of this bacterium in filarial disease manifestation and control, has caused a shift in the focus of disease mitigation towards indirect antifilarial therapy using antibiotics.

1.3. The discovery and rediscovery of *Wolbachia*

Wolbachia were first described (as intracytoplasmic bacteria) in the mosquito *Culex pipientis* (Hertig and Wolbach, 1924) and subsequently as unidentified intracellular organisms in *D. immitis*, *Brugia pahangi* (McLaren et al., 1975), *B. malayi* (Kozek, 1977) and *O. volvulus* (Kozek and Marroquin, 1977) fifty years later. However, it was the definitive molecular identification of *Wolbachia* in *D. immitis* in the 1990s (Sironi et al., 1995), which led to the realisation of the existence of *Wolbachia* in many filarial species (summarised in Table 1.1). This sparked renewed interest in the mechanisms of symbiosis, and the possibility of the development of human antifilarial therapeutics that work indirectly via the disruption of *Wolbachia* (Taylor and Hoerauf, 1999). It is hypothesised that some filarial species which do not harbour *Wolbachia*, including *Loa loa* (Buttner et al., 2003; Grobusch et al., 2003), *O. flexuosa* (Plenge-Bonig et al., 1995) and *Acanthocheilonema viteae* (Bandi et al., 1998) were once infected with *Wolbachia*, but suffered evolutionary loss along their respective lineages (Casiraghi et al., 2004) [Figure 1.3.1 (Bain et al., 2008). This is supported by the identification of *Wolbachia*-like DNA sequences within the filarial genome in the latter two filariae (McNulty et al., 2010). In the phylogenetic tree of the order Spirurida, the family Thelaziidae (outside the Filarioidea) and subfamilies Setariinae, Oswaldofilariinae and Waltonellinae (within the Filarioidea) are thought to have never been associated with *Wolbachia* (Figure 1.3.1). The practical significance of these differences is that it allows the comparison of, for example, the immunological responses of hosts infected with *Wolbachia*-dependent and

aposymbiotic filariae within *in-vivo* and *in-vitro* systems, and may reveal further clues as to the mutualistic functions of *Wolbachia*.

Table 1.1. Distribution of *Wolbachia* in filariae of medical, veterinary and comparative importance[†]

Filaria[*]	Natural host	Macrofilariae location	Microfilariae location	Reference (*Wolbachia*)
Wolbachia positive species				
O. volvulus	Human	Subcutaneous nodules	Skin	(Kozek and Marroquin, 1977)
O. ochengi	Bovine	Intradermal nodules	Skin	(Bandi et al., 1998)
O. armillata	Bovine	Aortic nodules	Skin	(Neary et al., 2010)
O. gutturosa	Bovine	Nuchal ligament	Skin	(Bandi et al., 1998)
O. lienalis	Bovine	Gastrosplenic omentum	Skin	
O. gibsoni	Bovine	Subcutaneous nodules	Skin	(Bandi et al., 1998)
O. tarsicola	Cervine	Subcutaneous tissue	Skin	(McLaren et al., 1975)
O. jakutensis	Cervine	Subcutaneous nodules	Skin	(Plenge-Bonig et al., 1995)
O. lupi	Canine	Ocular nodules	Skin	(Egyed et al., 2002)
B. malayi	Human	Lymphatics	Blood	(Kozek, 1977)
B. pahangi	Feline	Lymphatics	Blood	(McLaren et al., 1975)
W. bancrofti	Human	Lymphatics	Blood	(Bandi et al., 1998)
L. sigmodontis	Cotton rat	Pleural cavity	Blood	(Bandi et al., 1998)
D. immitis	Canine	Heart	Blood	(McLaren et al., 1975; Sironi et al., 1995)
Wolbachia negative species				
Loa loa	Simian	Subcutaneous tissue	Blood	(Buttner et al., 2003; McLaren et al., 1975)
O. flexuosa	Cervine	Subcutaneous nodules	Skin	(Plenge-Bonig et al., 1995)
A. viteae	Jird	Subcutaneous tissue	Blood	(Bandi et al., 1998; McLaren et al., 1975)
Species with unknown *Wolbachia* status				
O. ramachandrini	Porcine	Subcutaneous tissue	Limb skin	

[†] This table is not exhaustive; a recent publication has reported that many filarial species are probably devoid of *Wolbachia* (Ferri et al., 2011).
[*]Key to genus abbreviations: *O.*, *Onchocerca*; *B.*, *Brugia*; *W.*, *Wuchereria*; *L.*, *Litomosoides*; *D.*, *Dirofilaria*; *A.*, *Acanthocheilonema*.

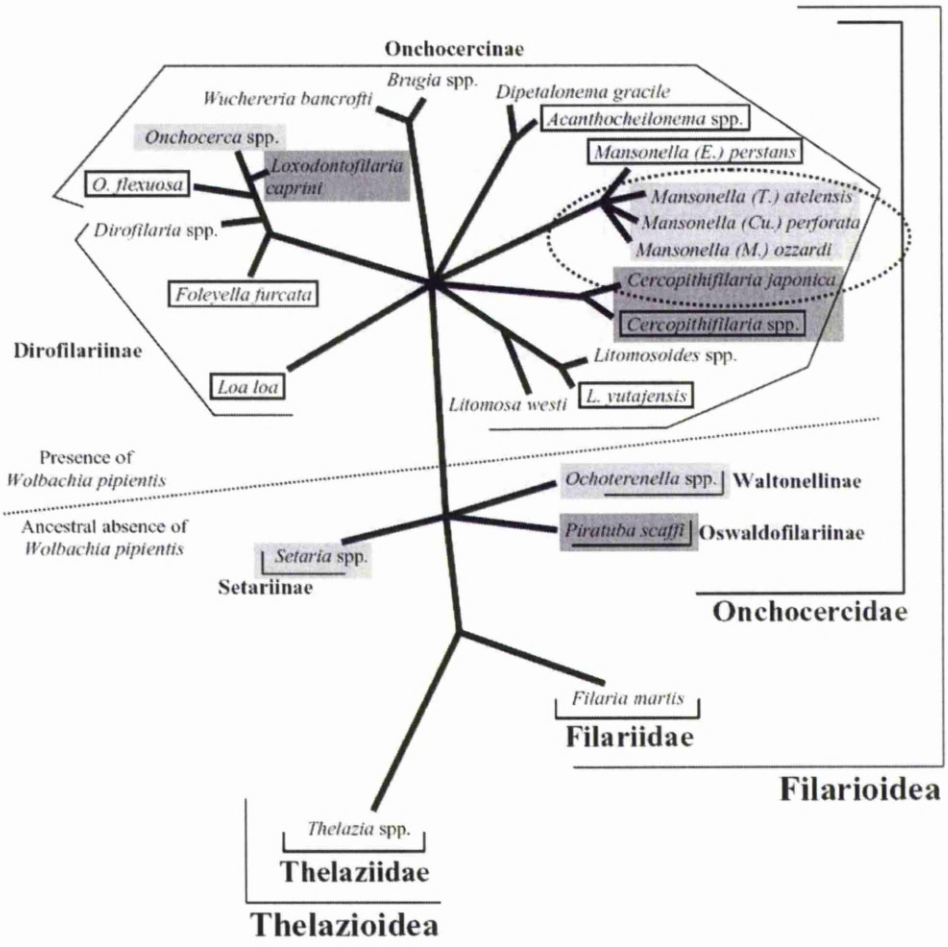


Figure 1.3.1. Phylogenetic tree of filariae (and related nematodes) overlaid with hypothetical evolution of *Wolbachia*. Species below the dotted line are hypothesised to have been ancestrally devoid of *Wolbachia*, whereas those above the dotted line are either *Wolbachia* replete, or are thought to have suffered secondary loss of *Wolbachia* (outlined in closed boxes). Onchocercinae and Dirofilarinae acquired *Wolbachia* subsequently classified as subgroups C and D. Species outlined with a dotted ellipse contain *Wolbachia* belonging to subgroup F. Dark grey shading includes species or genera studied since Casiraghi et al., (2004); light grey shading includes previously studies genera. Reproduced with kind permission from Bain et al., (2008) Parasite **15**, pp. 342-348.

1.4. The distribution of *Wolbachia*

Wolbachia are maternally transmitted alpha-proteobacteria of the family *Anaplasmataceae*, and are also widespread amongst insects, mites, spiders, scorpions and isopods. With an estimated two-thirds of all arthropod species infected (Hilgenboecker et al., 2008), including >10 million insect species (Werren et al., 2008), *Wolbachia* are thought to be the most abundant intracellular bacteria genus discovered. *Wolbachia* are transmitted vertically within host eggs, and as males are dead-end hosts, the evolutionary success of *Wolbachia* is attributed to its ability to manipulate host reproduction by a variety of mechanisms including feminization (Hiroki et al., 2002), parthenogenetic induction (Stouthamer et al., 1990), cytoplasmic (sperm-egg) incompatibility (CI) (Bourtzis et al., 1996), and even male killing. Although traditionally described as reproductive parasites of arthropods, there is evidence emerging of an evolutionary shift towards *Wolbachia* mutualism in the Californian fruit fly *Drosophila simulans*, which has experienced a natural spread of *Wolbachia* over the past two decades (Weeks et al., 2007). The shift, directed via *Wolbachia*-induced CI, has resulted in a positive change in insect fecundity, so that *Wolbachia*-infected female *D. simulans* exhibit a ten percent higher rate of egg production than uninfected females under laboratory conditions (Weeks et al., 2007).

In filariae, *Wolbachia* also confer mutualistic properties. Whilst they too are vertically transmitted via oocytes within the female host (Kozek, 1977), they do not induce a deleterious manipulative effect towards males, or a pathogenic effect towards either sex. However, their mutualistic role is apparent when they are removed from their host with antibiotic chemotherapy, as this inhibits larval development (Bosshardt et al., 1993), imparts filarial infertility (Hoerauf et al., 1999) and eventually causes worm death (Hoerauf et al., 2008; Langworthy et al., 2000).

The genomic sequencing of the *Brugia malayi Wolbachia* endosymbiont revealed a low density of functional genes compared to other bacteria, and elucidated considerable gene loss in metabolic pathways including those for many amino acids, biotin and folate (Foster et al., 2005). Reciprocally, *B. malayi* nuclear genome sequencing revealed loss of metabolic pathways for the synthesis of haem, riboflavin, and purines (Ghedin et al., 2007), which are retained in the symbiont, suggesting that *Wolbachia* engages in mutual exchange of nutrients with its host invertebrate (Foster et al., 2005; Slatko et al., 2010).

1.5. Contribution of *Wolbachia* to filarial disease

Wolbachia have been shown to contribute to the immunopathogenesis of filarial infections, not only during the lifetime of the parasite, but also after its death. For instance, in *O. volvulus* nodules containing live worms, *Wolbachia* contribute to the host neutrophilic response within onchocercomata (Brattig et al., 2001); and they cause the chemotaxis of neutrophils *in-vitro*, evidenced by reduced levels of the neutrophil chemoattractant IL-8 and impaired neutrophil chemotaxis in the presence of *Wolbachia*-depleted *O. volvulus* extracts (Brattig et al., 2001). In lymphatic filariasis, the desensitisation of mammalian host innate immunity in response to chronic low-level exposure to *Wolbachia*, via excretory/secretory (E/S) products and natural worm attrition, has been proposed to render the immune system vulnerable to opportunistic infection (Taylor et al., 2001). Lymphoedema, elephantiasis and subsequent dermatolymphangioadenitis are speculated to occur via this immune dampening process (Taylor et al., 2001). Indeed, the *in-vitro* desensitization of macrophages to extracts of *Brugia malayi* was shown to be dependent on *Wolbachia* (Turner et al., 2006). Furthermore, the observation of a reduction of plasma vascular endothelial growth factors (VEGF) was linked to a reduction in *Wolbachia*, and

preceded the amelioration of lymphoedema in doxycycline-treated lymphatic filariasis patients (Debrah et al., 2006).

Dead microfilariae are the primary cause of inflammation in the skin and the eye (Hall and Pearlman, 1999; Ottesen, 1995) and specifically, it is the release of *Wolbachia* which triggers innate and adaptive inflammatory responses (Taylor et al., 2005c). Neutrophils and eosinophils cause ocular pathology during experimental injection of filarial antigen into the murine conjunctiva (Kaifi et al., 2001), and it was found that *Wolbachia* directly activate neutrophils which then release cytokines and cause ocular pathology (Gillette-Ferguson et al., 2004).

Wolbachia also contribute to pathology after chemotherapy via their release from dying worms. In *O. volvulus*, post-treatment release of *Wolbachia* into the systemic circulation following DEC or ivermectin chemotherapy was found to positively correlate with clinical reaction scores and antibacterial peptides, with the highest levels of *Wolbachia* DNA detected (by PCR) following DEC treatment (Keiser et al., 2002). In addition, ivermectin-treated patients experienced an increase in systemic tumour necrosis factor alpha (TNF-α), which also correlated with an increase in *Wolbachia* DNA (Keiser et al., 2002). *Wolbachia* load was shown to be a significant factor in the severity of ocular pathology, as *O. volvulus* worms responsible for a severe form of ocular disease were found to contain higher *Wolbachia* loads than worms that caused the milder form (Higazi et al., 2005).

In patients infected with lymphatic filariasis, severe post-treatment reactions correlated with high microfilarial loads and the presence of whole *Wolbachia* cells in blood samples (Cross et al., 2001). Moreover, *B. malayi-Wolbachia* lipopeptide induced neutrophil-mediated keratitis (Turner et al., 2009), similarly to *O. volvulus*

extracts; but this contrasted with *Wolbachia*-depleted *O. volvulus* or the aposymbiotic filaria *Acanthocheilonema viteae*, where the inflammatory response was reduced or absent (Saint et al., 2002).

Wolbachia also play a role in the pathogenesis and immune response to infection with *D. immitis*. Anti-*Wolbachia*-surface protein (WSP) antibodies were detected after the experimental infection of immunologically naive cats (Bazzocchi et al., 2000) and naturally infected dogs (Kramer et al., 2005). Importantly, in the latter report, the antibodies detected were immunoglobulin G2 (IgG2), indicative of a bacterial-mediated T helper cell (Th) -1-biased response instead of a helminth-mediated Th-2 response. Necropsy findings revealed WSP within the lungs, liver and kidneys of dogs that had died from their infections (Kramer et al., 2005). Accordingly, when *D. immitis* infected dogs were treated with melarsomine, an intense inflammatory reaction within the lungs, together with WSP-positive staining similar to untreated controls, was observed (Kramer et al., 2008). In contrast, dogs that received a combination therapy of doxycycline and ivermectin had much less inflammatory-associated lung pathology and a decrease in WSP staining (Kramer et al., 2008), which is to be expected, as this drug combination was found to cause optimal *Wolbachia* depletion in *D. immitis* (Bazzocchi et al., 2008).

1.6. The continuing search to find effective anti-*Wolbachia* drugs

Although anthelmintics have traditionally been used to treat filarial infections, their lack of activity on *Wolbachia* can sometimes lead to adverse reactions (Hoerauf et al., 2003b). When used alone in the treatment of *D. immitis*, ivermectin did not exert anti-*Wolbachia* properties (Bazzocchi et al., 2008); however its use in combination with doxycycline resulted in a complete loss of *Wolbachia* (demonstrated via anti-

WSP staining). Although a depletory effect on *Wolbachia* was seen in the doxycycline only-treated group, the combination exhibited superior micro- and macrofilaricidal activity compared to either drug used alone, indicating a synergistic effect [*i.e.*, one drug potentiating the effect of the other (Bazzocchi et al., 2008).]

Tetracyclines are known to deplete *Wolbachia* in many filariae including *B. pahangi*, *D. immitis* (Bandi et al., 1999), *L. sigmodontis* (Hoerauf et al., 1999) and *O. ochengi* (Langworthy et al., 2000), where worm death in the latter was reported within 9 months post-treatment. As *Wolbachia* reside within the hypodermis, which is responsible for the production of the cuticle, the anti-*Wolbachia* effect of tetracycline was considered to exert an indirect effect on the worm cuticle, by blocking the L3 - L4 moult (Smith and Rajan, 2000). However, this was contradicted when a chemically-modified tetracycline with no antibacterial effects was shown to be more potent than conventional tetracycline at blocking the L3 - L4 moult in *B. malayi* cultures (Rajan, 2004).

One limitation when treating filariasis with tetracycline antibiotics is the length of treatment required. In *O. ochengi*, although a short intensive regimen (14 days) of oxytetracycline successfully depleted *Wolbachia*, the effect was only transient, and the recrudescence of *Wolbachia* by 24 weeks post-treatment ensured the continued viability of the adult worms. However, treatment with a longer, combined (COM) therapy (14 days initially, then monthly for 5 months) was sufficient to cause a sustained depletion of *Wolbachia* and a subsequent macrofilaricidal effect (Gilbert et al., 2005). Protracted treatment length is also required for macrofilaricidal effects in other filariases. Doxycycline exerts an antibacterial effect on *Wolbachia* leading to worm death in *W. bancrofti* (Debrah et al., 2007; Hoerauf et al., 2003a; Taylor et al.,

2005d), and *O. volvulus* (Hoerauf et al., 2001; Hoerauf et al., 2003b; Hoerauf et al., 2000a). With doxycycline chemotherapy, *W. bancrofti* can be killed within 3 - 4 weeks (Debrah et al., 2007), but the treatment length required for *O. volvulus* is 6 weeks (Hoerauf et al., 2003a). In *D. immitis*, prolonged intermittent treatment with doxycycline (up to 34 weeks), in conjunction with ivermectin, was found to exert complete macrofilaricidal and microfilaricidal effects (Bazzocchi et al., 2008). Hence, while the tetracycline antibiotics are undoubtedly effective antifilarial drugs that constitute a safer, slower-acting chemotherapy via the depletion of *Wolbachia* (that would otherwise contribute to mammalian host pathology), the length of treatment required, and their contraindications for use in small children and in pregnant and breastfeeding women, limit their application in practice. Despite this, the successful implementation of a community-directed delivery of doxycycline in an area co-endemic with *L. loa* (where the use of ivermectin is contra-indicated), illustrates that long treatment regimes can be practicable if effective distribution systems are established (Wanji et al., 2009).

In-vitro screening of the activity of minocycline on the motility and viability [via 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay] of *O. gutturosa* demonstrated an enhanced deleterious effect on *Wolbachia* relative to doxycycline (Townson et al., 2006). In addition, when compared to the effects of heat treatment on intermittent generations of *Trichogramma* wasps, minocycline was effective against *Wolbachia*, as evidenced by the interruption of thelytokous reproduction (*i.e.*, where females are produced from unfertilized eggs), a process which is dependent on *Wolbachia*. However, the wasps were never completely "cured" of *Wolbachia* (as thelytoky recovered after treatment), and the authors

reported that variable uptake of minocycline by the insects was the most probable explanation (Pintureau et al., 1999).

Rifampicin is a very effective anti-*Wolbachia* drug as determined by *in-vitro* screening, including adult *O. gutturosa* motility scores as an indicator of *Wolbachia* depletion (Townson et al., 2006), and both Giemsa-stained cytospin preparations (Hermans et al., 2001) and real-time PCR (Fenollar et al., 2003) in the *Aedes albopictus* (Aa23) mosquito cell line. When used for 2 or 4 weeks in the treatment of *O. volvulus in-vivo*, rifampicin significantly reduced the *Wolbachia* load (Specht et al., 2008), which remained depleted for 18 months post-treatment in the 4-week treatment group; although complete clearance was never achieved (in contrast to doxycycline). Combination therapy of rifampicin and doxycycline implemented over 14 days significantly depleted *Wolbachia* in the *L. sigmodontis* mouse model (Volkmann et al., 2003), indicating possible synergy via the complementary bacteriostatic (doxycycline) and bactericidal (rifampicin) effects.

Azithromycin could not deplete *Wolbachia* from *O. volvulus* in patients treated with 12 mg kg^{-1} for 5 days (Richards, Jr. et al., 2007), but was found to clear *Wolbachia* from adult *B. malayi in-vitro* when administered at 100 μg ml^{-1} for 10 days (Rao and Well, 2002). Erythromycin, trimethoprim and sulphamethoxazole were found to have no effect on filarial motility *in-vitro*, and MTT viability assays also suggested that these drugs are ineffective against *Wolbachia* (Townson et al., 2006). Direct quantification of *Wolbachia* using real-time PCR confirmed the inefficacy of erythromycin (Fenollar et al., 2003), chloramphenicol and ciprofloxacin (Hoerauf et al., 2000b). Penicillin and streptomycin (and the antimycotic amphotericin B) also had no deleterious effect on *Wolbachia* (or host filariae), as demonstrated *in-vitro*

with *B. malayi* (Rao and Well, 2002). In the Aa23 cell line, ciprofloxacin, ofloxacin and levofloxacin were reported to exert bacteriostatic properties, but their minimum inhibitory concentrations (MICs) were between 8 and 32 times higher than those of doxycycline or rifampicin (Fenollar et al., 2003). In the same study, MICs were also very high for gentamicin, thiamphenicol and cotrimoxazole in comparison with doxycycline and rifampicin; and penicillin G, amoxicillin and ceftriaxone showed no anti-*Wolbachia* effects whatsoever.

The ongoing search for novel chemotherapeutic targets has recently revealed that fosfomycin (Henrichfreise et al., 2009) and globomycin (Johnston et al., 2010) deplete *Wolbachia* in the *Aedes albopictus* C6/36wp cell line, via the interference of the biosynthesis of lipid products required for cell growth. Fosfomycin was found to interfere with lipid II biosynthesis, which is required as an intermediate for the synthesis of peptidoglycan [PDG (Henrichfreise et al., 2009)]; while the lipoprotein signal peptidase inhibitor globomycin was shown to affect the growth of *Wolbachia* in a dose-dependent manner via disruption of lipoprotein biosynthesis (Johnston et al., 2010). Although fosfomycin was not as effective at depleting *Wolbachia* as the tetracycline positive control (Henrichfreise et al., 2009), and globomycin had no detectable impact on *Wolbachia* load within *B. malayi in vitro* as determined by qPCR (Johnston et al., 2010), these studies have identified new obligate biochemical synthesis pathways within *Wolbachia* that may constitute novel chemotherapeutic targets. A summary of drugs and their effects on *Wolbachia* and host filariae is provided in Table 1.2.

Table 1.2. Drug activity against *Wolbachia* and effect on host filariae

Drug	System[♦]	Effect on *Wolbachia*	Antifilarial Activity[▲]	References
Antibiotics				
Oxytetracycline	*O. ochengi*	Depletion (COM therapy)	Micro/macrofilaricidal	(Gilbert et al., 2005) (Langworthy et al., 2000)
	Aa23 *(in-vitro)*	Depletion	-	(Hermans et al., 2001)
Tetracycline	*L. sigmodontis*	Depletion (50 mg/kg/day)	Filarial growth retardation/infertility	(Hoerauf et al., 2000b)
	B. pahangi	Depletion (24 -3 0 days treatment)	Blocking of embryo development	(Bandi et al., 1999)
	D. immitis			
Doxycycline	*W. bancrofti*	Depletion (200 mg/day for 8[†] or 6[††] weeks)	Micro/macrofilaricidal	[†](Taylor et al., 2005d) [††](Hoerauf et al., 2003a)
	D. immitis	Depletion (intermittently for 34 weeks)	Micro/macrofilaricidal [complete in combination with ivermectin (IVM)]	(Bazzocchi et al., 2008)
	O. volvulus	Depletion (100 mg/day for 6weeks)	Adult worm sterility	(Hoerauf et al., 2000a)
		Depletion (200 mg/day for 6 weeks)	Micro/microfilaricidal (more effective in conjunction with IVM)	(Hoerauf et al., 2008)
	Aa23 *(in-vitro)*	Depletion	-	(Hermans et al., 2001)
Rifampicin	Aa23 *(in-vitro)*	Depletion	-	(Hermans et al., 2001)
	O. volvulus	Depletion (10 mg/kg/day for 2 or 4 weeks)	Decreased embryogenesis and mff production	(Specht et al., 2008)
Erythromycin	*L. sigmodontis*	No effect	No effect	(Hoerauf et al., 2000b)
Chloramphenicol	*L. sigmodontis*	No effect	No effect	(Hoerauf et al., 2000b)
Ciprofloxacin	*L. sigmodontis*	No effect	No effect	(Hoerauf et al., 2000b)
	Aa23 *(in-vitro)*	No effect	-	(Hermans et al., 2001)
Penicillin	Aa23 *(in-vitro)*	No effect	-	(Hermans et al., 2001)
Penicillin	*B. malayi (in vitro*	No effect	No effect	

Streptomycin	cultures)	No effect	No effect	(Rao and Well, 2002)
Fosfomycin	*Aa*C6/36wp	Depletion	-	(Henrichfreise et al., 2009)
Globomycin	*Aa*C6/36wp	Depletion		(Johnston et al., 2010)
Anthelmintics				
Ivermectin	*D. immitis*	No effect	Degeneration of embryos. Macrofilaricidal (after 36 months chemotherapy)	(Bazzocchi et al., 2008)
	O. volvulus	No effect	Microfilaricidal	(Hoerauf et al., 2003b)
Suramin	*O. volvulus*	No effect	Macrofilaricidal	(Brattig et al., 2004b)
DEC	*W. bancrofti*	No effect*	Micro/macrofilaricidal	(Noroes et al., 1997)
	O. volvulus	No effect	Microfilaricidal	(Gutierrez-Pena et al., 1996)
Melarsomine	*O. ochengi*	Unconfirmed	Macrofilaricidal	(Tchakoute et al., 2006)

*No anti-*Wolbachia* effect inferred from *in-vitro* data reported (Gunawardena et al., 2005; Townson et al., 2006)
▲The observed reduction in microfilariae may occur secondarily to female sterilisation and attrition.
◆Key to genus abbreviations: *O.*, *Onchocerca*; *B.*, *Brugia*; *W.*, *Wuchereria*; *L.*, *Litomosoides*; *D.*, *Dirofilaria*; *A.*, *Acanthocheilonema*
Aa: *Aedes albopictus* mosquito cell-line

1.7. The bovine model for onchocerciasis

Molecular phylogenetic studies are consistent with the morphological and biological similarities between *O. volvulus* and *O. ochengi* (Xie et al., 1994), confirming the hypothesis that *O. ochengi* is the most closely related species to *O. volvulus*. This was originally proposed on the basis of their morphologic similarities [atrophic musculature and hypertrophic hypodermis of the adult female filariae of both species (Bain, 1981)]. *Onchocerca volvulus* and *O. ochengi* also share a common vector, *Simulium damnosum sensu lato* (Wahl et al., 1998a), in which their lifecycles are very similar (Figure 1.1.1). Indeed, an intriguing possibility is that the human parasite resulted from a host-switch by a subpopulation of the bovine worm, or that the two filariae speciated from a common ancestor during the domestication of cattle (Bain, 2002).

The integrity of the bovine model of onchocerciasis has been tested and validated via many *in-vivo*, *ex-vivo* and *in-vitro* experiments, and subsequently much is known about the dynamics of *O. ochengi* [reviewed in Trees et al (2000)]. Fortunately, bovine onchocerciasis is not associated with dermal or ocular disease, and the Mazzotti reaction (following DEC treatment) has not been observed in this species. The reasons for this are largely speculative, but could be due to lower skin microfilarial density (≤ 2 $\log_{10}$ lower than exhibited by the human counterpart), or a better adapted host response resulting in increased tolerance to infection (Trees et al., 2000). Thus, extensive longitudinal studies are ethically permissible in this model.

1.7.1. Chemotherapeutic investigations in the *O. ochengi* model

It has been demonstrated that chemotherapeutic agents do enter the *O. ochengi* nodule, and concentrations equivalent to or greater than serum are achieved (Cross et

al., 1997). Although a reduction in nodule diameter, adult worm numbers or male motility could not be achieved with prolonged avermectin chemotherapy (Bronsvoort et al., 2005), single or repeated high-dose treatment with ivermectin (Renz et al., 1995) and doramectin (Bronsvoort et al., 2005) caused sustained abrogation of embryogenesis and accumulation of dead microfilariae *in-utero*. Suramin, ivermectin and the compound 3-[(2-tert-butyl-5-methoxy-1, 3-benzothiazol-6-yl) carbamothioylsulfanyl] propanoic acid (CGP-20376) decreased skin microfilarial levels (over different timeframes), but in the suramin and CGP-20376-treated groups, microfilariae repopulated the skin. However, in the ivermectin-treated group, skin microfilarial levels dropped and remained low for the duration of the 137-day experiment (Renz et al., 1995). The same study showed that although intrauterine microfilariae were pathologically altered in all treatment groups, embryogenesis was only completely interrupted in the ivermectin and CGP-20376 –treated groups, whereas macrofilarial viability (as determined by MTT-formazan reduction assays) was not altered in any of the groups (Renz et al., 1995). The arsenical compound melarsomine, and the flubendazole derivative methyl (±)-[5-(α-amino-4-fluorobenzyl) benzimidazol-2-yl] carbamate (UMF-078), were then tested in the *O. ochengi* model, and were indeed found to be macrofilaricidal (Bronsvoort et al., 2008; Tchakoute et al., 2006). Unfortunately, UMF-078 is considered too toxic for human use; however, these trials provide further support for the applicability and relevance of the *O. ochengi* model.

When using long-term oxytetracycline therapy for the treatment of dermatophilosis in an experimental animal heavily-laden with *O. ochengi* nodules, the exciting and serendipitous observation was made that onchocercoma diameter gradually decreased, culminating in radical cure (Langworthy et al., 2000). After further

research into chemotherapeutic regimens of oxytetracycline, a short intensive treatment followed by a prolonged intermittent therapy (combined regimen) was found to be macrofilaricidal against *O. ochengi*, and subsequently this became the gold standard for macrofilaricidal chemotherapy in this model (Gilbert et al., 2005; Langworthy et al., 2000).

1.8. The genus *Onchocerca* and the filarial nodule

The genus *Onchocerca* encompasses approximately 30 species, and with the exceptions of *O. volvulus* and *O. lupi*, all are parasites of ungulates (Bain, 2002). In a minority of filarial species, the adult female worms remain encompassed within nodules which are formed either subcutaneously (as in *O. volvulus*), intradermally (as in *O. ochengi*), or in other anatomical loci (Table 1.1).

A schematic representation of the *O. volvulus* or *O. ochengi* archetypical nodule is shown in Figure 1.8.1. Within the fibrous capsule, adult female worms reside and reproduce (typically one adult female per nodule in *O. ochengi*, or ≥1 in *O. volvulus*). Male worms are migratory but visit the nodule to mate. Surprisingly, eosinophils, the effector cells most often associated with helminth infections, are never seen in direct contact with the filariae; they are scant, and found in the periphery of the cellular infiltrate (Wildenburg et al., 1996; 1997). Instead, female worms are surrounded by concentric rings of neutrophils in both *O. volvulus* (Brattig et al., 2001) and *O. ochengi* (Wildenburg et al., 1997), which are found lying directly adjacent to worm cuticles. Neutrophil migration towards adult female *O. volvulus* may be orchestrated by its E/S products (Rubio de Kromer et al., 1998), or towards the *Wolbachia* bacteria within the hypodermal chords and uterus of the worm (Brattig et al., 2001). Neutrophils are attracted directly by *Wolbachia* products such as WSP (Brattig,

2004a), and also indirectly by *Wolbachia*-induced production of cytokines including TNF-α and interleukin (IL) -8 from monocytes (Brattig et al., 2001). The accumulation of neutrophils around *Wolbachia*-containing worms may appear to be host-protective. However, the fact that the sessile adult worms are capable of flourishing for many years in this immunologically peculiar environment, where the neutrophils may contribute to a nutrient-rich cyst in which the anterior of the female worms is bathed, may indicate that these cells are actually parasite-protective (Brattig et al., 2001).

In *O. volvulus* nodules, it has been observed that those with female worms and microfilariae contain more eosinophils than those containing females without microfilariae, or just male worms alone (Wildenburg et al., 1996). Dead female worms contained within nodules are typically calcified and attract cells of chronic inflammation (*i.e.* macrophages and giant cells) within a lymphocytic infiltrate. Dead microfilariae may be associated with giant cells, or encompassed within small granulomas where they are typically surrounded by eosinophils, which adhere to the degenerating cuticle (Buttner and Racz, 1983). Disintegrating microfilariae may also be encased within microabscesses where neutrophils are present (Buttner and Racz, 1983). In *O. ochengi*, although there are more mast cells and eosinophils in nodules containing microfilariae-producing females, again they are never found adhered to the cuticle of adult worms (Wildenburg et al., 1997).

Histological studies of *O. flexuosa*, a nodule-forming filarial parasite of red deer, revealed nodule infrastructure similar to that of *O. volvulus* and *O. ochengi* (Plenge-Bonig et al., 1995). However, *O. flexuosa* does not contain *Wolbachia* and in contrast to *O. ochengi* and *O. volvulus*, the nodules it forms do not contain a

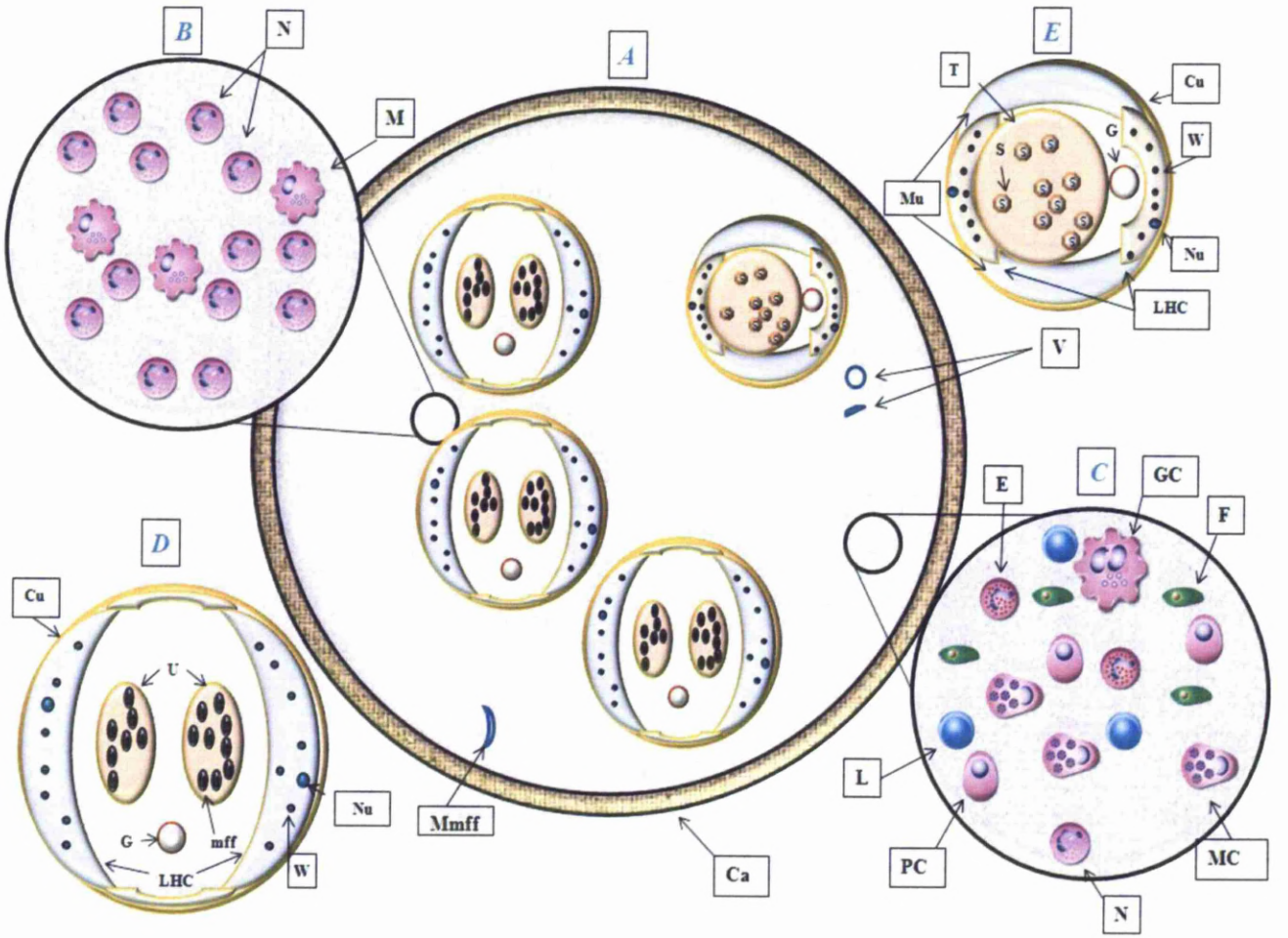
neutrophilic infiltrate (Brattig et al., 2001). Instead, *O. flexuosa* adult worms are surrounded by polarised giant cells that attack the cuticle (Brattig et al., 2001; Plenge-Bonig et al., 1995) and eosinophils, not neutrophils, are the predominant granulocyte observed in the nodule (Wildenburg et al., 1997). Interestingly, in this environment, *O. flexuosa* adult filariae appear to have an average lifespan of only ~1 year (Plenge-Bonig et al., 1995), in contrast to the 10 years or more reported for adult *O. volvulus* and *O. ochengi* (Duke, 1968).

Of the non-nodule-forming species, *Onchocerca gutturosa* is another *Wolbachia*-replete filaria of cattle. Adult worms are typically coiled and found in association with the lateral surface of the nuchal ligament (Wildenburg et al., 1997). Live female worms are usually surrounded by a 1.0 - 1.5 mm thick inflammatory cell infiltrate consisting of macrophages and giant cells in a palisade-like arrangement, followed by an outer layer of granulocytes, lymphocytes and plasma cells (Wildenburg et al., 1997). No cellular reaction has been detected around living male worms. In contrast to the nodule-forming filariae, neutrophils are not observed adjacent to the surface of the worms; instead they accumulate (together with eosinophils) around the outer layer of the cellular infiltrate, leaving a distinct space between the worm and the granulocytic periphery (Wildenburg et al., 1997). The surrounding tissue is highly vascular, and activated mast cells are present perivascularly and also in the outer inflammatory layer, where they are often associated with eosinophils (Wildenburg et al., 1997).

Thus, pertinent questions remain about the disparity of granulocytes within onchocercomata from different *Onchocerca* species, and the possible reasons for these differences. The apparent pattern of neutrophilic infiltration around nodule-

forming sessile worms is only seen in those species containing *Wolbachia*; and eosinophils, the cells most associated with parasitic helminth infections, are conspicuously sparse in untreated *O. volvulus* and *O. ochengi* nodules.

Figure 1.8.1. (following page): Schematic representation of a cross-section of a typical untreated *O. ochengi* or *O. volvulus* nodule (*A*). Inside are cross-sections of female (*D*) and male (*E*) filariae, migrating microfilariae (Mmff) and blood vessels (V), contained within the thick-walled capsule (Ca). The adult worms are protected by a cuticle (Cu), and internally are flanked by lateral hypodermal cords (LHC) containing nuclei (Nu) and *Wolbachia* (W). The pseudocoelomic cavity contains a small gut (G). The adult female worm (*D*) is distinguishable by the paired uteri (U) containing various stages of developing microfilariae (mff); and the male (*E*) is distinguishable by the hypertrophic musculature (Mu) required for migration, and testes (T) containing various stages of spermatozoa (S). Cellular infiltrates (*B*) adjacent to adult worms consist of many neutrophils (N), with scattered macrophages (M). This contrasts with the cellular environment towards the periphery of the nodule (*C*) where there are fewer worm sections. This cellular infiltrate is mixed, and consists of giant cells (GC), fibroblasts (F) plasma cells (PC), lymphocytes (L), mast cells (MC), eosinophils (E), and (infrequently) neutrophils (N). Images of cells were reproduced from the Academy of Immunology for Clinicians (copyright-free material) [http://www.aic-belgium.net/_en/images-bank-1]


B
N
M
A
E
T
Cu
S
G
W
Mu
Nu
LHC
V
C
GC
E
F
L
PC
MC
N
D
Cu
U
G
mff
LHC
W
Nu
Mmff
Ca

1.9. The structure and function of human and bovine granulocytes

1.9.1. The neutrophil

Neutrophils are the predominant circulating leukocyte in man (60 - 75% of all circulating leukocytes), and the second most abundant circulatory leukocyte in ruminants and laboratory rodents (~20 - 30%) after the lymphocyte. Following differentiation and development from pluripotent stem cells within the bone marrow [at a rate of 10^{11} cells per day in the healthy adult (Athens et al., 1961)], mature neutrophils are released in to the circulation, where they either reside within the circulatory pool, or become sequestered in the microvasculature (marginal pool). After 12 hours in the circulatory system, neutrophils enter the tissue, where in the healthy individual, they live for a few days before programmed cell death (apoptosis) ensues.

Neutrophils are essential effector cells of the innate immune system. Their primary function is defined by the toxic substances stored within the vast number of membrane-bound granules present within the cytoplasm. Early electron microscopy studies on rabbit neutrophils identified two major types of granules: primary (or azurophilic) and secondary (or specific) granules (Bainton and Farquhar, 1966), which are also identified in human neutrophils. Azurophilic granules are larger, synthesized in the promyelocyte, and contain products which impart antimicrobial and antiviral effects (e.g. defensins) and cytotoxic properties, such as myeloperoxidase, lysozyme, cationic antibacterial proteins and acid hydrolases (Rausch and Moore, 1975). Myeloperoxidase (MPO) is an essential factor in the respiratory burst mechanism of the neutrophil, catalysing the reaction between hydrogen peroxide (resulting from the dismutation of superoxide anions) and intracellular halides to form bactericidal hypohalides, and imparts the green

colouration to pus. However in bovines, the respiratory burst is influenced by arachidonic acid (Aebischer et al., 1993). Lysozyme exerts a lytic action on Gram-positive bacteria via hydrolysis of the cell wall PDG (Baggiolini, 1972), but can only reach the deeper layer of PDG in the wall of Gram- negative bacteria after modification of the outer membrane by other granule products [papain, elastase and cathepsin G (Thorne et al., 1976). Bovine neutrophilic granules lack lysozyme (Padgett and Hirsch, 1967); however, they were shown to actively ingest *Staphylococcus aureus*, and exert antibody-dependent cell-mediated cytotoxicity *in-vitro* (Roth and Kaeberle, 1981).

Specific granules are smaller and are formed only in the myelocyte. They contain lysozyme, lactoferrin, collagenases, gelatinases, histaminases and vitamin B12-binding protein. Elastases and collagenase facilitate the migration of neutrophils through the extracellular matrix, and can cause tissue injury during inflammation. Lactoferrin binds iron necessary for bacterial growth (Paape et al., 2003), and also increases neutrophil adherence.

Bovine neutrophils also contain a third type of granule, which is larger, denser, and more numerous than the peroxidase-positive azurophil or specific granules, and these are released independently of azurophils (Gennaro et al., 1983). 'Large' granules contain at least two types of the highly bactericidal, cationic polypeptides termed bactenecins (Zanetti et al., 1990), which were found to exert potent antimicrobial activity *in-vitro* (Selsted et al., 1992). Bovine neutrophils (and eosinophils) contain a PDG recognition protein named bovine oligosaccharide-binding protein; the mature protein is stored in the cytoplasmic granules and was found to be microbicidal for

both Gram-positive and -negative bacteria (where PDG is buried), yeast, and also towards bacteria in which PDG was absent (Tydell et al., 2002).

In addition to their predominantly phagocytic role, neutrophils help to perpetuate the proinflammatory immune response by the production of TNF-α (Bazzoni et al., 1991a), IL-1 (Goto et al., 1984), platelet activating factor (PAF) and LTB_4, (McDonald et al., 1993). They can also produce IL-8 during phagocytosis, establishing a positive-feedback mechanism whereby further neutrophils are recruited (Bazzoni et al., 1991b). More recently, it was demonstrated that neutrophils incubated with T-cells or their cytokines acquired major histocompatibility complex (MHC) class II antigens (Hansch et al., 1999), and were able to present antigen to T-cells in a MHC class II-restricted manner in both humans (Iking-Konert et al., 2001) and bovines (Whale and Griebel, 2009). Thus, bovine and human neutrophils are similar in their function of bacterial clearance, albeit with subtle mechanistic and granule content differences, which are summarised in table Table 1.3.

1.9.1.1. Neutrophil recruitment

The primary role of neutrophils is concerned with first-line defence of the mammalian body, and as such neutrophils are the first cells to arrive at a site of tissue insult or inflammation. The recruitment and trafficking of neutrophils from the marginal pool to the target site, and their interaction with specific membrane receptors, requires a sophisticated multifaceted sequence of events (Butcher, 1991). The presence of a high concentration of a chemoattractant within an area of the vascular endothelium induces the initial and reversible endothelial interaction (rolling) via selectins (transmembrane glycoproteins), which may be rapidly expressed by Complement 5a [C5a (Foreman et al., 1994)] or histamine (Jones et al.,

1993). Platelet selectins [P-selectins (Burns et al., 1999)] and endothelial selectins [E- and L-selectins (Lawrence et al., 1994) play an important role in tethering neutrophils by binding with their counter-ligands P-selectin glycoprotein-1, or E-selectin ligand-1, respectively (Diacovo et al., 1996). Firm, non-reversible endothelial adhesion follows via the interaction of integrins [neutrophils predominantly express the cluster of differention (CD)-18 integrin], which bind to intracellular adhesion molecules (ICAM)-1 and 2, expressed on the endothelial cell surface (Springer, 1990). Subsequent transcellular or paracellular diapedesis enables the neutrophil to leave the circulation and enter the tissues (Kvietys and Sandig, 2001).

1.9.1.2. Chemotaxis and neutrophilic chemokines

Chemotaxis (directional) and chemokinesis (non-directional), describes the movement of a granulocyte in the presence of a stimulatory gradient of a chemical substance. Classical human neutrophilic chemoattractants include products C3a and C5a of the complement cascade (Fernandez et al., 1978), LTB_4 (Ford-Hutchinson et al., 1980), certain chemokines such as IL-8 (Baggiolini et al., 1989), and *N*-formylmethionyl-leucyl-phenylalanine (fMLP) peptides of bacterial origin (Schiffmann et al., 1975).

The fundamental importance of chemotaxis is demonstrated by the success of notoriously virulent bacteria. For example, supernatant from *Staphylococcus aureus* cultures was found to abrogate chemotaxis towards C5a and fMLP via the downregulation of their neutrophil receptors; however the activity of IL-8 was not affected (Veldkamp et al., 2000). Interestingly, bovine neutrophils do not have fMLP receptors and thus do not migrate towards this peptide (Brown and Roth, 1991), but

during *S. aureus* mastitic infection, neutrophils were found to migrate towards IL-8 (Barber and Yang, 1998). In lipopolysaccharide (LPS)-induced mastitis, high concentrations of C5a/C5a^{desArg} are present in neutrophil-rich milk; whilst C5a present in normal milk may provide the chemoattractant necessary for a baseline migration of neutrophils into the non-mastitic udder (Paape et al., 2003).

Chemokines are key mediators of chemotaxis (Baggiolini and Dahinden, 1994; Godaly et al., 2001), but also play a role in the process of degranulation, synthesis of lipid mediators, and the activation of integrins (Baggiolini et al., 1997; Laudanna et al., 2002; Springer, 1994). Chemokines are small (8 - 14 kDa) basic proteins that are divided into two main groups based on the position of their first two cysteine residues, which are either adjacent (CC chemokines) or separated by a single amino acid [CXC chemokines (Zlotnik and Yoshie, 2000)]. Lymphotactin, a C chemokine possessing two instead of four cysteines (Kelner et al., 1994), and fractalkine, a CX_3C chemokine possessing 3 amino acids between the first two cysteines (Bazan et al., 1997), are atypical structures. Further division of the CXC chemokines into ELR+ and ELR- groups (based on the presence or absence of the tri-amino acid ELR motif 'Glu-Leu-Arg', which precedes the first cysteine residue at the amino terminal), classifies the potent human neutrophil chemoattractants IL-8 (CXCL8), growth-related oncogene alpha [GRO-α (CXCL1)], GRO-β (CXCL2) and epithelial neutrophil activating peptide-78 [ENA-78 (CXCL5)] as ELR+ CXC chemokines. In contrast, the ELR- CXC chemokines include SDF-1α (CXCL12) and IP-10 (CXCL10), which act predominantly on lymphocyte populations [reviewed in Gale and McColl (1999)].

Interleukin-8 (8.4 kDa in the human) is potently proinflammatory and may be present in both chronic and acute reactions [reviewed in Hoch et al (1996)]. IL-8 can induce neutrophil extravasation (Huber et al., 1991), degranulation (Willems et al., 1989), and the respiratory burst (Peveri et al., 1988). IL-8 can signal via CXCR1 and CXCR2 receptors; however, only the former is specific for this chemokine (Ahuja et al., 1996; Baggiolini et al., 1997). IL-8 causes the release of lactoferrin from the large granules in bovine neutrophils; this is important in coliform mastitis, as the binding of iron by lactoferrin prevents its uptake by Gram-negative bacteria (Paape et al., 2003). IL-8 is a major chemoattractant of neutrophils during mastitis, and also causes subsequent release of alkaline phosphatase from secondary granules (Paape et al., 2003). Recombinant bovine IL-8 attracted neutrophils (but not eosinophils) in a dose-dependent manner *in-vivo*; and *in vitro* was found to induce neutrophil chemotaxis and shape-change (Caswell et al., 1999). More recently IL-8 production by endometrial cells in response to metritis caused by bovine herpesvirus has been reported (Donofrio et al., 2010); where the authors suggested that the influx of neutrophils into the post-partum endometrium may be a novel antiviral mechanism, or alternatively may help to curtail secondary bacterial infection.

In humans, the molecular structures of GRO-α and IL-8 are similar, and as such both chemokines bind to the CXCR2 receptor (Baggiolini et al., 1997). *In-vitro*, GRO-α and IL-8 have been shown to be equipotent in their neutrophil chemoattractant properties (Balentien et al., 1990), although they were also found to be functionally distinct, as GRO-α acted indirectly via the induction of IL-8 and TNF-α (Fujiwara et al., 2002). GRO α, β and γ (CXCL1, 2 and 3, respectively) all bind to the CXCR2 receptor. In bovines, GRO γ has been identified as the primary neutrophil

chemoattractant of non-mastitic milk, and is secreted from mammary epithelial cells within the normal udder (Rainard et al., 2008).

Epithelial cell-derived neutrophil-activating peptide-78 (ENA-78) is a potent chemoattractant and activator of neutrophils, with similar biological properties to the GRO family and IL-8 in humans (Walz et al., 1991). ENA-78 was first shown to be secreted from IL-1β or TNF-α stimulated epithelial cells (Walz et al., 1991), but is also produced by eosinophils (Persson et al., 2003), monocytes (Schnyder-Candrian and Walz, 1997) and mast cells (Lukacs et al., 1998). In cattle, the bovine homologue of ENA-78 (boENA-78) has been identified (via immunohistochemistry) in alveolar epithelial cells from pneumonic bovine lungs, and shown to exert similar neutrophilic chemotactic and activation properties when secreted from pulmonary monocytes and macrophages *in-vitro* (Allmann-Iselin et al., 1994)

1.9.2. **The eosinophil**

Eosinophils serve as multifunctional effector cells of the innate branch of the immune system of mammals, and are characterized by their granular uptake of acidic dye (eosin). The bilobed nucleus may be partially obscured by granules, which fill the cytoplasm and constitute the armoury of the cell. Eosinophils participate in the immune response elicited by a broad range of diseases, including allergy, inflammatory disorders and viral infections (Phipps et al., 2007), but are perhaps most noted for their role in helminthiases, where they exhibit enhanced cytotoxicity and release inflammatory mediators (Klion and Nutman, 2004). Eosinophils are produced in the bone marrow from myeloid stem cells similarly to neutrophils, and undergo differentiation before IL-5-mediated release into the circulation (Collins et

al., 1995), where they comprise only 1 - 2% of blood leukocytes in humans (Rankin et al., 2000), but up to 20% in the adult bovine (Lumsden et al., 1980).

Eosinophils contain distinct granule types, the morphology and constitution of which varies between species (Table 1.3). Specific (secondary) granules are first detectable within the myelocyte. The distinguishing feature of human and rodent eosinophils is a crystalloid core which contains major basic protein [MBP (Miller et al., 1966)]. Bovine specific granules contain very low amounts of MBP (Duffus et al., 1980) and accordingly, bovine eosinophils lack the crystalline core evident in other species. However, they do possess a similar protein that contains relatively high concentrations of lysine and thiol, which has been shown to kill juvenile *Fasciola hepatica in-vitro* (Duffus et al., 1980). MBP carries an extreme positive charge, and exerts hydrophobic properties associated with cytotoxicity. When in contact with biological membranes, MBP increases permeability via modification of the surface charge, and binds to anionic domains on cells and parasites, resulting in disruption of the lipid bilayer (Wasmoen et al., 1988). The central MBP core is surrounded by a matrix, containing the ribonucleases eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN). ECP is also helminthotoxic (McLaren et al., 1981) and bactericidal via the perforation of the target cell plasmalemma with non-selective pores (Gleich and Loegering, 1984; Lehrer et al., 1989). Eosinophil peroxidase (EPO), which is involved in the respiratory burst in eosinophils, catalyses the oxidation of halides (Agosti et al., 1987), pseudohalides and nitric oxide (Wu et al., 1999). Interestingly, bovine eosinophils have been reported to contain up to 5 times more peroxidase than human eosinophils, and the oxidative burst in bovine eosinophils was found to be quantitatively similar to that of bovine neutrophils [(as assayed by nitroblue tetrazolium reduction) (Roth and Kaeberle, 1981)].

Primary granules contain hexagonal Charcot-Leyden crystals that interact with lysophospholipases (Ackerman et al., 2002). They are present in tissues and secretions infiltrated by eosinophils and basophils, and are characteristic signs of allergy (Dor et al., 1984). Human and rat eosinophils also contain 'small' granules, which appear to be involved in microendocytosis(Komiyama and Spicer, 1975). Small granules contain arylsulphatase B, acid phosphatase and possibly catalase, and are upregulated in certain disease states (Parmley and Spicer, 1975).

1.9.2.1. Eosinophil chemotaxis and activation

Similar to neutrophils, eosinophils undergo chemotaxis from the systemic circulation via a process of rolling, adhesion and transendothelial migration mediated by selectins, integrins and other adhesion molecules (Rothenberg and Hogan, 2006). These are up-regulated by the Th-2 cytokines IL-4 (Fukuda et al., 1996) and IL-13 (Bochner et al., 1995), and the chemokines eotaxin and 'regulated upon activation, normal T cell expressed and secreted' (RANTES). The cytokine IL-5 increases the responsiveness of mature eosinophils to degranulation and respiratory burst (Kita et al., 1992) and to chemotaxis mediators. IL-5 acts by binding to specific receptors on the surface of B cells and eosinophils, and prolongs the life of the eosinophil via the inhibition of apoptosis (Stern et al., 1992). IL-5 together with eotaxin shows synergistic properties with respect to the mobilisation of eosinophils from the bone marrow pool (Rankin et al., 2000), and when administered intravenously, it acts synergistically with intradermally administered eotaxin to facilitate eosinophil accumulation in the skin (Collins et al., 1995). In the guinea pig, IL-5 mediates localised eosinophil accumulation *in-vivo* (Collins et al., 1995; Iwama et al., 1992) and *in-vitro* (Coeffier et al., 1991).

The chemokine eotaxin [8.4 kDa in humans (Rankin et al., 2000)] is a potent, selective CC chemokine which is produced by epithelial and endothelial cells, and also by eosinophils themselves (Garcia-Zepeda et al., 1996). It binds to the CCR3 receptor, which is highly expressed on eosinophils, but poorly expressed on basophils and Th-2 cells (Rankin et al., 2000), and absent on neutrophils and monocytes (Garcia-Zepeda et al., 1996). The murine homologue of eotaxin is also directly chemotactic for eosinophils, and is strongly induced in response to interferon-γ (IFN-γ) *in-vitro* (Rothenberg et al., 1995). In the bovine, recombinant eotaxin was found to attract eosinophils in a concentration-dependent manner *in vitro*, but was not found to be of physiological importance in the facilitation of eosinophil migration in the ovary (Vogel et al., 2005). This function is instead exhibited by RANTES, which was found to be expressed by ovarian macrophages and mediates an alternative route of physiological eosinophil chemotaxis during the pre-ovulatory period (Aust et al., 1999). Eotaxin upregulates the CD11b integrin subunit, thus enhancing the adhesion of eosinophils to endothelial cells, and stimulates the adhesion of eosinophils to human TNFα-activated endothelial cells (Burke-Gaffney and Hellewell, 1996). Eotaxin also potently activates the respiratory burst in human eosinophils (Elsner et al., 1996).

Eosinophils synthesize, store and secrete RANTES, which in common with eotaxin, can cause differential secretion of cytokines (IL-4 but not IL-12) from eosinophils via piecemeal degranulation (Moqbel and Coughlin, 2006).

Table 1.3. Characteristics of cytoplasmic granules in human and bovine neutrophils and eosinophils

Granulocyte	Human	Bovine	References
Neutrophil			
	1° azurophil granules: larger; contain MPO, lysozyme, cationic antibacterial proteins, acid hydrolases	1° azurophil granules: granules do not contain lysozyme	(Padgett and Hirsch, 1967; Rausch and Moore, 1975)
	2° specific granules: smaller; contain lysozyme, lactoferrin	2° specific granules	
		3° cationic granules: contain "bactenecins"; more numerous than other granules; 0.4 - 0.5 μm; peroxidase-negative; round profile with more dense matrix; lack peroxidase, acid hydrolase, neutral proteinases, but contain lactoferrin; also contain bovine oligosaccharide-binding protein	(Zanetti et al., 1990) (Gennaro et al., 1983) (Tydell et al., 2002)
Eosinophil			
	1° granules: contain Charcot-Leyden crystals		
	2° granules: MBP crystalline core; periphery of ECP, EDN, EPO	2° granules: round or elongated, very dense, non-crystalline core; granule diameter 0.5 - 0.8 μm; low concentration of MBP (normal major component of crystalline core); high concentration of EPO	(Duffus et al., 1980)
	'Small granules': arylsulphatase; acid phosphatase; catalase		(Roth and Kaeberle, 1981)

Key to granule proteins: MPO, myeloperoxidase.; MBP, major basic protein.; ECP, eosinophil cationic protein.; EDN, eosinophil derived neutrotoxin.; EPO, eosinophil peroxidase.

Table 1.4. Key human and bovine granulocyte chemotactic cytokines.

Cytokine	Sources	Receptor	Bovine comparisons/contrasts
Neutrophilic			
IL-8 (CXCL8)	Monocytes, endothelial cells	CXCR1 (specific) CXCR2 (non-specific)	
GRO α (CXCL1) GRO β (CXCL2)	Epithelial cells, macrophages, neutrophils Monocytes, macrophages	CXCR2	
GRO γ(CXCL3)	Prostate epithelial cells (Kogan-Sakin et al., 2009)	CXCR1/CXCR2	Produced by mammary epithelial cells in normal milk (Rainard et al., 2008)
ENA-78 (CXCL5)	Epithelial cells, eosinophils, mast cells, monocytes, macrophages	CXCR2	Homologue boENA-78 expressed similar properties to human cytokine (AllmannIselin et al., 1994)
Eosinophilic			
Eotaxin-1 (CCL11)	IFN-γ-stimulated endothelial cells, TNF-α-activated monocytes Activated monocytes, T-cells	CCR3	Belongs to the MCP/eotaxin subfamily based on different chimeric structure (Vogel et al., 2005)
Eotaxin-2 (CCL24)	IL-4 or IL-13-stimulated endothelial cells	CCR3	
Eotaxin-3 (CCL26)	Mast cells, T-cells, eosinophils	CCR3	
IL-5	Th-2 cells, mast cells, eosinophils (Dubucquoi et al., 1994), endothelial cells, epithelial cells, smooth muscle cells	CCR3/CCR4/CCR5	Bovine IL-5 shares 55% amino acid sequence identity with human IL-5 (Mertens et al., 1996)
RANTES (CCL5)	Epithelial cells (Taguchi et al., 1998), $CD8^+$ memory T-cells (Walzer et al., 2003), eosinophils (Moqbel and Coughlin, 2006)	CCR1/CCR2/CCR3/CCR5	Expressed in ovarian macrophages (Aust et al., 1999)

†Cytokine key: IL, interleukin.; GRO, growth regulated oncogene.; ENA, Epithelial cell-derived neutrophil-activating peptide.; RANTES, regulated upon activation, normal T-cell expressed and secreted.; IFN, interferon.; TNF, tumour necrosis factor; MCP, monocyte chemoattractant protein.

1.10. Toll-like receptors

Toll-like receptors (TLRs) are conserved from insects to humans (Anderson, 2000), with homologues playing a vital role in antifungal immunity in *Drosophila* (Lemaitre et al., 1996). They have been described as key elements of innate immunity, where they are critical for the detection of pathogens and the initiation of an appropriate immune response (Aderem and Ulevitch, 2000). TLRs are prominently expressed in antigen presenting cells, including dendritic cells (Kadowaki et al., 2001), macrophages (Underhill et al., 1999), and also granulocytes (Hayashi et al., 2003; Nagase et al., 2003). To date, thirteen TLRs have been identified (TLR1 - 13) and most of these (a key exception is TLR-3) are expressed by human neutrophils (Hayashi et al., 2003); while a smaller subset (TLR-1, 3, 4, 7, 9 and 10) has been identified in human eosinophils (Mansson and Cardell, 2007; Nagase et al., 2003). An overview of the expression of TLRs in granulocytes and their respective ligands is presented in Table 1.5.

In-vitro, TLR-2 has been shown to recognise bacterial lipoproteins by directly binding to them (Vasselon et al., 2004) and subsequently causing immune stimulation such as apoptosis and activation of the respiratory burst (Aliprantis et al., 1999). TLR-4 recognises lipid A, a component of lipopolysaccharide (LPS) from Gram-negative bacteria (Hirschfeld et al., 2000). TLR-5 recognises flagellin and TLR-9 identifies bacterial CpG DNA (Akira et al., 2001), but only after phagocytosis, as TLR-9 is only expressed in intracellular vesicles (Ahmad-Nejad et al., 2002).

An outer membrane protein from *Neisseria meningitidis*, the TLR-2 ligand PorB, was found to specifically induce the recruitment of eosinophils into the peritoneum

of mice via a Th-2 skewed response, and was suggested for use as a vaccine adjuvant where the eosinophilic clearance of helminths is the desired outcome (Burke et al., 2007). Eosinophils were found to express a greater quantity of TLR-7 than neutrophils, which together with TLR-8, was shown to regulate the expression of adhesion molecules CD11b and L-selectin, prolonging the survival of and inducing superoxide generation in eosinophils (Nagase et al., 2003). TLR-7 was found to mediate eosinophil degranulation *in-vitro* when activated by respiratory syncytial virus ssRNA in cultures of murine splenocytes (Phipps et al., 2007).

1.10.1. Toll-like receptors in the bovine

Homologues of all TLRs except TLR-6 have been identified in bovine skin, and the most abundant were TLR-4 and TLR-7 (Menzies and Ingham, 2006). TLR-2 and-4 mRNA was locally up-regulated in tissues collected from mastitic udder quarters, in contrast to uninfected quarters removed from the same cow (Goldammer et al., 2004). Surprisingly, both TLRs were also up-regulated in udders monoinfected with *Staphylococcus aureus*, suggesting that even during a purely Gram-positive infection, TLR-4 works in tandem with TLR-2 (Goldammer et al., 2004). Although they were not isolated in this experiment, neutrophils are the primary cells associated with bovine mastitis, thus the increase in TLR-2 and 4 probably occurred in these cells.

1.10.2. Toll-like receptors and *Wolbachia*.

Bacterial proteins can be potent stimulators of innate inflammatory responses (Henderson et al., 1996). More specifically, WSP was found to activate neutrophils via their TLR-2 and -4 receptors, and in the absence of TLR-4, the inflammatory response induced by WSP was diminished (Brattig et al., 2004b). *In-vitro*, TLR-2

and -6 receptors recognised *Wolbachia*-derived diacylated lipoprotein (which was dependent on both the lipid and protein moieties) and *in-vivo*, synthetic *Wolbachia* lipopeptide induced neutrophil-mediated corneal inflammation via TLR-2/6 pathways in the murine keratitis model (Turner et al., 2009). In the same model, TLR-2 was also found to mediate CXC chemokine production by corneal bone-marrow derived cells (Gillette-Ferguson et al., 2007). In the mouse chamber model, TLR-4 was found to play a role in protective immunity against larval *O. volvulus* L3, but surprisingly, not via a response to *Wolbachia* (Kerepesi et al., 2005).

Table 1.5. Overview of the Toll-like receptors expressed on human and bovine granulocytes, and their respective ligands

TLR	Ligands	Granulocyte expression (human)	Granulocyte expression (bovine)
TLR-1	Triacylated lipoproteins	Eosinophils (Nagase et al., 2003)	
TLR-2	Lipoproteins Yeast zymosan Mycobacterial ara-lipoarabinomannan Lipotechoic acid	Neutrophils (Kurt-Jones et al., 2002)	
TLR-3	Viral dsRNA (Mansson and Cardell, 2007)	Eosinophils* (Nagase et al., 2003)	
TLR-4	Heat-shock proteins Lipopolysaccharide Viruses C5a	Eosinophils (Nagase et al., 2003)	Neutrophils (Stevens et al., 2011; Worku and Morris, 2009)
TLR-5	Flagellin	Neutrophils [but primary expression in monocytes (O'Mahony et al., 2008)].	
TLR-6	Diacylated lipoproteins	Neutrophils (Nakao et al., 2005)	
TLR-7	Viral ssRNA (Lund et al., 2004; Phipps et al., 2007)	Eosinophils (Nagase et al., 2003; Phipps et al., 2007)	
TLR-8	Viral ssRNA	Neutrophils (Yanagisawa et al., 2009)	
TLR-9	CpG oligodeoxynucleotides	Neutrophils (O'Mahony et al., 2008); Eosinophils (Nagase et al., 2003)	
TLR-10	**Unknown**	Eosinophils (Nagase et al., 2003)	

*Expressed on murine cells.

1.11. The immune response to filarial disease

1.11.1. Mechanisms of parasite killing

In general, host responses to helminth infections are remarkably similar (Maizels et al., 1993), dominated by a Th-2 response which is characterized by the production of IL-4, IL-5, IL-9, IL-10 and IL-13. This milieu leads to eosinophil mobilisation, mast cell accumulation and the production of IgE (Macdonald et al., 2002), which acting together, can mediate parasite destruction as evidenced with *Strongyloides stercoralis in-vitro* (Rotman et al., 1996). Clearance of gastrointestinal nematodes can also occur via IL-13-directed overproduction of mucus (McKenzie et al., 1998).

The human immune response to *O. volvulus* infection is similarly dominated by elevated IgE, a peripheral eosinophilia, and the production of IL-4 and IL-5 by peripheral blood mononuclear cells [PBMCs (King and Nutman, 1991)]. Indeed, eosinophils have been found to play an essential role in filarial killing by a process of chemotaxis, activation and degranulation, causing irreversible damage to the surface of the parasite (Greene et al., 1981; Johnson et al., 1991). Eosinophils have been implicated in the immobilisation of *O. volvulus* L3 in the presence of immune serum *in-vitro*, where they migrated towards the larvae and within hours, formed layers in which cell adherence and degranulation occurred, with release of EPO (Brattig et al., 1991). Eosinophils were also found to degranulate on or near the cuticular surface and cause death of the L3 before completion of their moult *in vitro* (Strote et al., 1990).

Degranulating eosinophils have been observed around damaged or dead *O. volvulus* microfilariae *in-vivo* (Ackerman et al., 1990), within lymph vessels containing degenerating microfilariae (Racz et al., 1982) following treatment with DEC, and

within lymph nodes of *O. volvulus* patients following ivermectin treatment (Wildenburg et al., 1994). After DEC treatment, both eosinophils and neutrophils actively attacked the surface of damaged microfilariae, and the neutrophils released granule products such as elastase, MPO and lysozyme; while the eosinophils released EPO, which was also considered to contribute towards microfilarial death (Gutierrez-Pena et al., 1996). Despite exerting a host-protective role in helminthiases, eosinophils can also contribute towards host pathology via tissue infiltration and degranulation of non-target-specific cytotoxic granule proteins, as observed in some patients with sowda (hyperreactive onchocerciasis) and lymphatic filariasis (Klion and Nutman, 2004).

In the *O. lienalis* murine model, T-cell facilitated microfilarial clearance was achieved (Carlow et al., 1988), and resistance to re-infection was shown to be Th-2 dependent (Folkard and Bianco, 1995). Eosinophils were found not only to be of key importance (in comparison to macrophages and neutrophils) in facilitating the clearance of a primary infection, but also in protective immunity against re-infection; and in both cases, IL-5 was critical for mediating these responses (Folkard et al., 1996). Martin et al. demonstrated IL-5-mediated eosinophilia and immune-mediated protection in *L. sigmodontis* L3-vaccinated BALB/c mice, reporting a reduction in incoming larvae by 70% within two days post-infection; the effect of which was abolished when using IL-5 knockout mice (Martin et al., 2000b). A similar scenario was reported in the mouse chamber model, where immunity to *O. volvulus* L3 (implanted in subcutaneous diffusion chambers) was found to be dependent on both eosinophils and IgE (Abraham et al., 2004). Incidentally, as murine eosinophils do not express IgE receptors, alternative pathways of eosinophil recruitment involving complement, or indirect signalling via mast cells were inferred (Mayr et al., 2002).

More recently, the complex nature of the immune response toward filarial infection has become apparent. Studies of the immunological pathways associated with onchocerciasis in cattle have revealed an immune response that is not strongly Th-2-biased. After experimental inoculation of L3, the early Th response was non-polarized, but was followed by down-regulation of initial peaks in both IL-4 and IFN-γ when the infection reached patency (Graham et al., 2001). In mice and humans, T-regulatory (T-reg) cells have been connected with the down-regulation of the classical Th-2 pathway (Doetze et al., 2000; Taylor et al., 2005a), and have been isolated from the nodules of human patients with few dermatological signs (Satoguina et al., 2002). During L3 infection of the *B. pahangi* mouse model, a T-reg response was shown to modulate Th-2 cytokines via the production of IL-10 and expression of TGF-β mRNA, and this was independent of IL-4 (Gillan and Devaney, 2005). Furthermore, *L. sigmodontis* infections reached patency concurrently with Th-1 and Th-2 dampening, which was directed by T-reg cells; and the survival of *L. sigmodontis* was attributed to the hypo-responsive state induced by this subset (Taylor et al., 2005a).

The role of the recently discovered Th-17 lineage of cells revealed their contribution toward the counter-regulation of the T-reg milieu, via the cytokines IL-17, IL-21 and IL-23, in patients suffering with filarial lymphoedema (Babu et al., 2009). Th-1 responses were also significantly enhanced, which accompanied an increased expression of TLR-2, 4 and 7 in the neutrophils of these patients (Babu et al., 2009).

Neutrophils are strongly associated with the Th-1 and Th-17 response, and Th-17 cells are integral in the establishment and maintenance of a range of chronic inflammatory diseases [reviewed in Ouyang et al. (2008)]. Th-1 and Th-17 pathways

are also associated with the up-regulation of vascular endothelial growth factors from fibroblasts, which may exacerbate pathology (Numasaki et al., 2004). Neutrophils, via release of elastase and other cytotoxic products, contribute towards a variety of adverse systemic reactions after routine ivermectin treatment for onchocerciasis (Njoo et al., 1993). Neutrophils are also associated with murine *O. volvulus*-induced keratitis, where they were found to migrate via binding of platelet endothelial cell adhesion molecule [PECAM-1 (Kaifi et al., 2001)]. However, neutrophils have also been found to mediate filarial killing. For instance, in the presence of immune serum, neutrophils can kill *O. volvulus* microfilariae (Johnson et al., 1991); and in the mouse model, neutrophils also accumulated around *L. sigmodontis* L3 (Martin et al., 2000a).

1.11.2. **Filarial mechanisms of immune evasion**

The various host immune responses that may counteract filarial infection have been discussed thus far; however, fundamentally the success of filarial parasites serves as a marked illustration of the failure of the host to overcome infection. Filariae of all stages have developed mechanisms of immune evasion to ensure survival. In mice injected with *L. sigmodontis* (Le Goff et al., 2000b) and *Loa loa* (Wanji et al., 2002), a significant proportion of L3 avoid immune attack by entering lymphatic vessels. In addition, *in-vitro* studies on *O. volvulus* revealed that after moulting, the distinct cuticular morphology of the L4 stage seems to enable the abrogation of eosinophil adherence (Brattig et al., 1991; Lustigman et al., 1990). Adult *B. malayi* synthesises a glutathione peroxidase (Maizels et al., 2001) to protect its cuticle from the damaging effects of free oxygen radicals by reducing phospholipid hydroperoxides (Tang et al., 1994). Vitamin E, an important constituent of the filarial cuticle recognised as possessing potent antioxidant properties, is acquired by

filariae from the host's extracellular fluid (Smith et al., 1998); and thioredoxin peroxidase (TPX), a member of the peroxiredoxin superfamily, protects from oxidative stress by detoxifying host hydrogen peroxide. TPX is contained in multiple stages of *O. volvulus*, and may aid in parasite transmission as its expression is greatly up-regulated in infective larvae (Lu et al., 1998).

Brugia malayi, *W. bancrofti* and *O. volvulus* secrete a homologue of the human proinflammatory cytokine, macrophage migration inhibitory factor (MIF), which is capable of inhibiting monocyte and macrophage migration by up to 75%, and affecting chemotaxis in an identical way to human macrophage migration inhibitory factor [MIF (Pastrana et al., 1998)]. *O. volvulus* also contains onchocystatin, which is thought to modulate cysteine proteases and initiate cellular hyporeactivity via the up-regulation of IL-10, which can inhibit cell-mediated responses (Lustigman et al., 1992).

Apart from the various physical and chemical methods of immune evasion exhibited by filarial worms, a unique characteristic that sets the sessile, nodule dwelling filariae *O. volvulus* and *O. ochengi* apart from other filariae is the neutrophilic environment in which the adult worms are bathed throughout their long life. It has been previously demonstrated that this highly unusual environment is due to the presence of *Wolbachia*, because after the depletion of the endobacteria from *O. volvulus* via doxycycline chemotherapy, the neutrophilic environment recedes (Brattig et al., 2001). Furthermore, the aposymbiotic, sessile, nodule-forming *O. flexuosa* is never associated with a neutrophilic infiltrate (Brattig et al., 2001) and importantly, a much shorter lifespan for this worm has been suggested (Plenge-Bonig et al., 1995). However, eosinophils *can* become activated and will migrate

towards worms normally replete with *Wolbachia* if the symbionts are targeted by antibiotics. This was elegantly demonstrated in an experiment by Nfon et al, who showed that the infiltration of eosinophils into the neutrophilic environment within *O. ochengi* nodules was concurrent with the depletion of *Wolbachia* in cows receiving a non-macrofilaricidal, short intensive regimen (SIR) of oxytetracycline (Nfon et al., 2006). Crucially, following the SIR, *Wolbachia* recrudesced (Gilbert et al., 2005) in synchrony with a reversal of the cellular environment towards the heavily neutrophilic milieu. At this time, the eosinophils were excluded once more, which suggested that the eosinophils had infiltrated as a result of the neutrophilic decline that followed *Wolbachia* depletion (Nfon et al., 2006). However, although eosinophils were seen to degranulate in the vicinity of oxytetracycline-treated worms, this happened over a prolonged post-treatment period. Thus, it was unknown whether they were performing an active cytotoxic role, or formed part of a secondary wound healing response alongside macrophages to clear organic debris *after* filarial death (Nfon et al., 2006).

The gaps in our knowledge regarding the role of *Wolbachia* in filarial survival are worthy of further investigation. It has been hypothesized that *Wolbachia* may be responsible for the long reproductive lifespan of adult worms due to a continuous supply of nutritional factors (Slatko et al., 2010). However, it is unknown whether *Wolbachia* play a further role in immune evasion via an ability to skew the innate immune response towards a more favourable neutrophilic environment within worm nodules, in order to protect the worm host from eosinophil degranulation. This fascinating question is the subject of this thesis.

2. Study approach

2.1. Hypothesis and experimental design

Previous studies could not conclusively determine whether eosinophils are a cause or a result of filarial morbidity (Nfon et al., 2006) This study aims to further test the hypothesis that neutrophil accumulation around nodule-dwelling filariae occurs as a consequence of *Wolbachia*, which in a novel example of defensive mutualism, confers filarial longevity through immune evasion. Thus, if this hypothesis is correct, removal of the neutrophil population (via antibiotic chemotherapy targeting *Wolbachia*) is a necessary precedent for filarial killing by eosinophil degranulation.

A longitudinal *ex-vivo* study of nodules extirpated from naturally infected Cameroonian cattle enabled analysis of chronological changes in *Wolbachia* populations, worm viability and granulocyte dynamics collectively for the first time. Cattle were treated with macrofilaricidal regimens of oxytetracycline or melarsomine, and nodulectomies were conducted over a 54-week period. Prior verification that melarsomine lacked antibiotic effects against *Wolbachia* was determined using an *in-vitro* viability study of *Wolbachia*-infected Aa23 mosquito cells. Concurrent *in-vitro* cell migration assays using purified bovine granulocytes in the presence of *Wolbachia*-replete or -depleted homogenised worm samples were performed to elucidate direct interactions between *Wolbachia* and granulocytes.

Ex-vivo changes in nodules were investigated by means of cytological quantification and differentiation via light and electron microscopy; *Wolbachia* distribution using immunohistochemistry; and the quantification of bacterial and filarial viability indicators [16S rRNA and *O. ochengi* glutathione S-transferase (*Oo*-GST)

transcripts, respectively), alongside bovine cytokine expression (IL-8, IL-5, GRO protein family and eotaxin), using qRT-PCR.

2.2. Objectives of the study

- To confirm the transition of the cell population of *Onchocerca ochengi* nodules from predominantly neutrophils, to eosinophils in coincidence with anti-*Wolbachia* chemotherapy (oxytetracycline).
- To determine that the cellular transition from neutrophils to eosinophils in *O. ochengi* nodules is specific for anti-*Wolbachia* (oxytetracycline) chemotherapy.
- To verify in an *in-vitro* system that melarsomine has no direct affect on *Wolbachia*.
- To investigate the specific cytokine signalling responsible for the cellular changes related to anti-*Wolbachia* chemotherapy in the *O. ochengi* nodule.
- To investigate the process of filarial killing by eosinophils.

3. Materials and methods

3.1. Evaluation of the effect of melarsomine on the viability of *Wolbachia*

3.1.1. Rationale and experimental design

The Aa23 cell-line, derived from the mosquito *Aedes albopictus*, is persistently infected with *Wolbachia pipientis* (O'Neill et al., 1997) from the B supergroup. It provides an *in-vitro* system for reproducible experiments involving the systemic manipulation of *Wolbachia* that would not be feasible using filarial cell cultures due to their failure to immortalise (Higazi et al., 2004). In addition, a range of drugs has been evaluated within this system using Giemsa staining and microscopy (Hermans et al., 2001), qPCR (Fenollar et al., 2003), or qRT-PCR (Makepeace et al., 2006). Doxycycline exhibits a minimum bactericidal concentration of 0.25 μg ml^{-1} against *Wolbachia* (Hermans et al., 2001) but has no toxic effect on Aa23 cells (Makepeace et al., 2006), thus providing an ideal positive control for anti-*Wolbachia* activity in this system. The qRT-PCR assays for rRNA developed by Makepeace et al. (2006) have the advantages of detecting only viable (or recently killed) organisms and allowing for normalisation against host cell transcripts.

3.1.2. Cell culture and treatment

Stock Aa23 cells in cryopreservation medium (Appendix 12.8.1) were resuscitated from liquid nitrogen storage by incubation at 37°C in a water bath until defrosted. All subsequent procedures were performed in a laminar flow cabinet. Cells from each vial were re-suspended in growth medium (Appendix 12.8.1) in a final volume of 5 ml, and centrifuged at 150 *g* for 10 minutes at 20° C. The supernatant was

removed and replaced with 5 ml of fresh growth medium, and the cell suspension was transferred into a 25 cm^2 cell-culture flask (Nunc). The cells were incubated at 26°C in air, and the medium was replaced after 24 hours to remove cells that failed to adhere. Once the cells had reached approximately 80% confluence, they were sub-cultured by dislodging the monolayer with a cell scraper, diluting the suspension 1/5 with maintenance medium (see Appendix 12.8.1), and dividing the cells equally between new flasks.

Doxycycline hyclate (Fluka) and melarsomine hydrochloride (a kind gift from C. Marcato, Merial, Toulouse) were dissolved in ultrapure water and sterilised by filtration (0.2 μm). Flasks were treated in quadruplicate at three drug concentrations (Table 3.1) and harvested after 3 or 7 days post-treatment. Untreated control cells received an equivalent volume of sterile water and were harvested in parallel.

Treatment	**Concentration (μg ml^{-1})**
Doxycycline hyclate	0.04
	0.1
	0.25
Melarsomine dihydrochloride	0.04
	0.1
	0.25
Control (sterile water)	0

Table 3.1 Drug concentrations used during treatment of the Aa23 insect cell-line

3.1.3. RNA extraction, cDNA synthesis and qRT-PCR

Aa23 cells were harvested from the flasks and subsequently processed using the protocol documented in Makepeace et al (2006), with the following modification.

Prior to cDNA synthesis, contaminating DNA was removed from the RNA by the addition of 2 U TURBO DNase in 1× TURBO DNase buffer (Ambion) in a final volume of 20 µl, and incubated for 30 minutes at 37°C. The DNase was deactivated using 2 µl DNase deactivation reagent (Ambion), which was incubated for 2 minutes at room temperature before removal by centrifugation at 10,000 *g* for 90 seconds.

Quantitative RT-PCR reactions for *Wolbachia* 16S rRNA and *A. albopictus* 18S rRNA were conducted on a MiniOpticon Real-Time PCR Detection System (Bio-Rad) using custom primers [www.biomers.net (Ulm, Germany)] and oligonucleotide standards (Sigma) based on nucleotide sequences detailed in Makepeace et al (2006). Samples were assayed at several concentrations to ensure that copy numbers were interpolated from the central region of the standard curves, using reaction conditions as previously reported (Makepeace et al., 2006). In parallel, no-template control (nuclease-free water) and reverse transcriptase (RT)-negative samples were run to confirm the specificity of the assays. The values for RT- reactions were negligible (<1 copy) in 83% of cases; for the remaining samples, the RT+ copy numbers were corrected by subtraction of the corresponding RT- value prior to statistical analysis.

3.1.4. Statistical analysis

Statistical analysis was conducted using PASW statistics version 17 software (SPSS Inc., Chicago, IL, USA). Data were normalised by $\log_{10}$ transformation, and Kolmogorov-Smirnov tests (with exact significance) were used to confirm that the transformed data did not deviate significantly from a normal distribution. A full factorial general linear model (GLM) was employed using type III sum-of-squares and an intercept, with drug and concentration as fixed factors. Post-hoc analyses

were performed for drug and concentration using Dunnett's post-hoc *t*-test (two-tailed), defining a drug concentration of zero as the reference control group.

3.2. *In vivo* experiment

3.2.1. Animal sourcing and experimental conditions

All procedures performed on cattle in Cameroon were equivalent to those previously authorised by a Home Office Project Licence [Animals (Scientific Procedures) Act 1986] for experimental infections of cattle in the UK, and classified under the severity limit of 'mild'.

The experiment was carried out at the IRAD Regional Centre at Wakwa, Ngaoundéré, North Cameroon; a cattle farming area of Guinea Savannah forming part of the Adamawa Province. In this area of Cameroon, the prevalence of bovine onchocerciasis is geographically patchy (Wahl et al., 1998b). Within the Adamawa Plateau, areas close to the fast-flowing River Vina (a significant breeding site for *Simulium damnosum*) have a high prevalence of *O. ochengi*, and cattle grazed on pasture at this site are exposed to up to 400 L3 per day (Wahl et al., 1998a) during optimal conditions (*i.e.*, the dry season). Conversely at IRAD, located some 10 km from the River Vina, the prevalence of *O. ochengi* and its transmission potential are negligible (Tchakoute et al., 2006). This situation facilitated the purchase of naturally infected animals that could be kept at IRAD without the likelihood of re-infection.

Eighteen healthy Gudali (*Bos indicus*) cattle (fifteen cows and three bulls), infected with *O. ochengi* and laden with >30 palpable nodules each, were purchased from nearby markets and kept at pasture at the field site for the duration of the experiment. During their time at IRAD, two female animals died from causes unrelated to the experiment; thus, any data collected from these animals whilst alive was excluded from subsequent analyses.

3.2.2. Cattle grouping, nodule mapping and chemotherapy

Cattle were ear-tagged, weighed, and divided into three groups each containing five cows (ear tags numbers were randomly selected using a statistical calculator) and one bull. Bulls were included in this experiment due to the difficulty in finding sufficient heavily-infected cows at market. Nodules were identified by examination of the skin, lifting and rolling it between the thumb and index finger, and applying slight pressure to differentiate from demodicosis. Nodules were most abundant on the ventrum (specifically around the udder/testes), inguinum, and lateral neck. The periphery of each nodule was tattooed using veterinary tattoo ink (Cox®) with a 25 G hypodermic needle, and their locations were recorded on 'hide maps' (Appendix 12.1) and in digital video clips. Nodule pairs were randomly selected for removal at planned time-points (Appendix 12.3). Additional tattooed (but unpaired) nodules were mapped for extirpation for electron microscopy studies (Appendix 12.3).

Group name	No. animals	Drug	Regimen
OXY	6	Oxytetracycline (Terramycin® LA, Pfizer)	10 mg kg^{-1} IV once daily for 14 days; 14 day break, then 20 mg kg^{-1} IM monthly for 5 months
MEL	6	Melarsomine (Cymelarsan®, Merial)	4 mg kg^{-1} IV every other day for a total of 3 treatments.
CON	6	None	None

Table 3.2. Experimental design

Cattle received chemotherapy according to Table 3.2. The "gold standard" of antibiotic chemotherapy for *O. ochengi,* which kills 80% of adult worms by twelve months after the start of oxytetracycline treatment (Gilbert et al., 2005), was followed. For intravenous (IV) drug administration, cattle were either placed in sternal (OXY group) or lateral (MEL group) recumbency. Melarsomine was used as

a non-antibiotic control, as it is 100% adulticidal (Tchakoute et al., 2006) and directly targets nutrient uptake and metabolism in the worm (Subrahmanyam, 1987). Cymelarsan® (Merial) was reconstituted to a concentration of 5 mg ml^{-1} using sterile water (Lonza). Each dose was decanted into a sterile, empty intravenous drip bag (Kruuse) and administered IV over ten minutes via a catheter attached to a 3-way tap and 50-ml syringe. Control animals received no treatment.

3.2.3. Nodule extirpation and gross processing

The course of worm death in animals receiving the oxytetracycline regime occurs over approximately one year (Gilbert et al., 2005), with a change in intranodular cellular composition occurring by 8 to 12 weeks (Nfon et al., 2006). To capture a range of chronological 'snapshots' of intranodular dynamics throughout the year-long experiment, nine time points were selected for nodulectomies. These were at 0, 2, 4, 8, 12, 24, 36, 48 and 54 weeks post-treatment (wpt).

At each time point, cattle were gathered in a corral and placed in lateral recumbency (Appendix 12.4). Pre-determined tattooed nodule pairs were identified (Appendix 12.3), the local hair clipped, and the skin aseptically prepared. Nodule pairs were surgically removed (Appendix 12.4) under local anaesthesia (Willcain®, Dechra Veterinary Products) and the wounds sutured. At every other time point a third nodule was extirpated for electron microscopy studies. Post-operation sites were checked daily and summer fly cream (Battle, Hayward & Bower Ltd) applied as necessary to prevent myiasis. At the corral, nodules were placed in phosphate buffered saline [(PBS) Sigma] for transport to the laboratory, where they were trimmed of excess tissue.

At the field-site laboratory, nodules intended for histopathological studies were immersed and injected with 10% neutral-buffered formalin for fixation prior to storage and transportation at ambient temperature. Nodules intended for electron microscopy were immersed and injected with 5% glutaraldehyde solution in 75 mM sodium cacodylate buffer (pH 7.4) prior to storage and shipping at 4°C. Nodules intended for qRT-PCR analysis were injected with RNA*later*® (Sigma), weighed, and incubated in 5 volumes of RNA*later*® overnight at 4°C, then stored at -20°C. Prior to transport, nodules were cut into 1 mm^3 pieces and homogenised in 12 volumes of Lysis-Binding solution [RNAqueous®-Midi Kit (Ambion)] as per the manufacturer's instructions, using an IKA® Ultra Turrax® homogenizer fitted with an IKA® 8mm stainless steel dispersing element. Homogenates were shipped to the UK on dry ice and stored at -80°C.

3.2.4. Nodule histopathology and granulocyte quantification

Nodules were bisected using a scalpel blade, embedded in paraffin, and 4 µm sections were cut from the centre using a microtome before mounting on glass slides. Mounted sections were deparaffinised and rinsed in descending concentrations of ethanol, and finally water, before staining with a 1/45 dilution of Giemsa in distilled water for one hour. Sections were then rinsed in distilled water and differentiated in a 1/1,500 dilution of glacial acetic acid for 30 seconds, rinsed again, and dehydrated using 95% ethanol, 100% ethanol, and xylene before mounting using DPX. To ensure that the sections were examined "blind" by the primary investigator (RDEH), the original serial numbers were obscured before assorting the slides into an arbitrary sequence and relabeling them. Using an Eclipse 80i microscope (Nikon UK, Kingston-upon-Thames) fitted with a DS-U1 camera control unit and NIS-Elements Basic Research 3.0 software, images were obtained of whole nodule sections at ×2

magnification. These were digitally 'stitched' so that the circumference and diameter of each nodule could be measured. A horizontal midsection transect of each nodule was then captured digitally at ×20 magnification, and the images stitched to create a panorama, which was subsequently divided into three equal sections and overlaid with a digital grid. Neutrophils and eosinophils were differentiated and counted by eye, tagged using the computer software, and normalised to cells per mm^{-2}. Each horizontal section image was scanned visually for degranulating eosinophils which were then semi-quantified using a four-point scale (0 = 0, 1 = <1, 2 = 1 – 10, 3 = >10 per worm section).

3.2.5. Nodule immunohistochemistry

The histopathological studies indicated that the intranodular cellular composition was at its most dynamic between 8 and 24 weeks post-oxytetracycline treatment (Section 6.2.1). Thus, subsequent analyses were focussed on the time-points of 8, 12 and 24 weeks post-treatment.

For immunohistochemistry, nodule sections were deparaffinised in xylene (two incubations of 5 minutes each, with change of solvent) and slowly rehydrated in 100% ethanol followed by 95% ethanol (two incubations of 3 minutes each per concentration), and then transferred into distilled water for 30 seconds. Antigens were demasked by incubating the sections in 0.1 M sodium citrate buffer, pH 6.0 (Appendix 12.8.2), for two periods of 5 minutes, with a change of buffer. Slides were air dried and a hydrophobic ring drawn around each section (Dako Pen, Dako) before applying reagents from a LSAB2 System-Horseradish Peroxidase (HRP) kit (Dako). Briefly, each section was incubated with peroxidase block reagent (Dako) for 5 minutes, rinsed in distilled water, and incubated in BSA-sucrose blocking

buffer (Appendix 12.8.2) for 30 minutes. Following a brief rinse in wash buffer (Appendix 12.8.2), the slides were incubated with a rabbit polyclonal anti-*Wolbachia* surface protein antibody (α-WSP) at a 1/500 dilution in blocking buffer for 30 minutes. This antibody was raised against recombinant WSP from *Dirofilaria immitis* (Kramer et al., 2003). For each section incubated with α-WSP, an adjacent section was identically processed using a 1/500 dilution of normal rabbit serum (Sigma) as a negative control. Slides were rinsed and incubated with biotinylated link reagent (Dako) for 10 minutes, rinsed again, and streptavidin-HRP (Dako) was applied for 10 minutes. Positive reactivity was detected following incubation with 3,3'-diaminobenzidine (DAB) chromogen (Dako) for 5 minutes, and excess substrate was removed with distilled water. Finally, the slides were counterstained with Giemsa (Section 3.2.4), air dried and mounted using DPX.

Sections were examined at ×40 magnification using a Leica Dialux compound microscope (Wetzlar, Germany). As DAB chromogen leaves a brown precipitate when bound to its target antigen-antibody complex, the presence of *Wolbachia* could be detected in adult worm tissues and in any microfilariae present in the nodule. The slides were then analysed via a two-step screening process. Firstly, the overall state of the nodule including integrity, presence of microfilariae, and the distribution and relative quantities of *Wolbachia* was evaluated, and the abundance of neutrophils and eosinophils was scored on a scale of 1 to 3. Secondly, a digital zonal scale was centred over each cluster of >10 eosinophils, and their proximity to *Wolbachia* precipitate and worm structures was semi-quantified in each of three 50-micron zonal increments, then recorded using a table (Appendix 12.5), which was adapted from a form proposed by Striebel (1988).

3.2.6. Transmission electron microscopy

Glutaraldehyde-fixed nodules were dissected and 1 - 2 mm^3 blocks containing *O. ochengi* were washed in 0.1 M cacodylate buffer and then placed in 1% aqueous osmium tetroxide for 90 minutes. Fixed tissue blocks were stained using 8% uranyl acetate in 0.69% maleic acid for 90 minutes, then dehydrated in ascending ethanol concentrations (50, 70, 90 and 100% with two incubations of 5 minutes each); finishing in 100% acetone (three 10-minute incubations with change of solvent). Resin (TAAB Laboratories Equipment) infiltration was accomplished by placing blocks in solutions of 30% and 60% resin in acetone once, then in 100% resin twice, for 2 hours each. Tissue blocks were then embedded in 100% resin, before incubating at 60°C overnight for polymerisation to occur. Blocks were cut into 0.5 µm semi-thin sections, stained with toluidine blue and checked for structural integrity using a light microscope, before cutting ultrathin (60 nm) sections using a Reichert-Jung Ultracut ultramicrotome equipped with a diamond knife (Diatome Ltd, Biel, Switzerland). Ultrathin sections were mounted onto 200-mesh copper grids and stained with Reynolds' lead citrate for 8 minutes. Digital micrographs were taken using an H7100 transmission electron microscope (Hitachi High Technologies, Krefeld, Germany) fitted with a charge-coupled device camera (Hamamatsu, Massy, France) and digital image acquisition software (Advanced Microscopy Techniques, Danvers, MA, USA).

Definitive TEM reference images of bovine neutrophils and eosinophils were created from fresh bovine cells separated using the protocol detailed in Section 3.3.3.1. Neutrophil and eosinophil-rich cell pellets were fixed in 5% glutaraldehyde in 0.1M sodium cacodylate buffer, then processed for TEM as outlined above. Digital images were acquired using a Philips EM 208s transmission electron microscope (Philips)

equipped with Gatan digital imaging, and bovine neutrophils and eosinophils differentiated according to their ultrastructural characteristics (Appendix 12.6).

3.2.7. Quantitative reverse transcriptase PCR on nodular material

3.2.7.1. RNA extraction and cDNA synthesis

RNA extraction and purification by lithium chloride precipitation was performed using the RNAqueous®-Midi Kit (Ambion), following the manufacturer's instructions. Briefly, homogenised nodules were thawed and centrifuged at 21,900 *g* for 8 minutes at 4°C. The clarified lysate was removed and an equal volume of 64% ethanol was added before passing the lysate-ethanol mixture through a glass filter. Each filter containing the RNA was washed using Wash Solutions 1 and 2, and the RNA was recovered by flushing the filters with Elution Solution (0.1 mM EDTA) heated to 100°C. Lithium Chloride Precipitation Solution (0.5 volumes) at 4°C was added to each sample prior to incubation for 30 minutes at -20°C, followed by centrifugation at 21,900 *g* for 15 minutes. The resultant RNA pellet was washed with 70% ethanol and re-centrifuged as before. The ethanol was removed and the pellets suspended in 100 µl hot Elution Solution prior to storage at -80°C.

The RNA content of the eluates was quantified using a NanoDrop®-1000 spectrophotometer (Thermo Fisher Scientific), and if required, the samples were concentrated by centrifugation for 8 minutes at 5,000 *g* in Nanosep® Omega 30 kD filtration units (Pall Life Sciences). The RNA content was verified and all samples were normalised to ~3 µg in 17 µl nuclease-free water. All incubations were performed on a T3 thermocycler (Biometra). To ensure complete solubilisation of RNA, samples were incubated for 10 minutes at 55°C, and contaminating DNA was digested by addition of 1 µl (2 U) of TURBO DNase (Ambion) with 2 µl TURBO

DNase buffer, followed by incubation for 30 minutes at 37°C. The DNase was deactivated by addition of 2µl DNase Inactivation Reagent (Ambion) and incubation for 2 minutes at room temperature, before removal of the reagent gel by centrifugation at 10,000 *g* for 90 seconds. Reverse transcription proceeded using a SensiMix™ Two-Step Kit (Quantace), following the manufacturer's protocol. Firstly, to enable annealing, the supernatant from each RNA sample was mixed with random hexamers (final concentration, 10 µM) and dNTP (final concentration, 1 mM) and incubated in a final volume of 20 µl at 65°C for 10 minutes, then chilled on ice for 2 minutes. Secondly, the samples were split into two equal aliquots and combined with 50 U reverse transcriptase (RT+ aliquot), or an equivalent volume of nuclease-free water (RT- aliquot), and 10 U RNase inhibitor in 1× SensiMix™ RT buffer (final volume, 20 µl). Finally, to obtain first-strand cDNA, the samples were incubated at 40°C for 50 minutes (extension) and 70°C for 15 minutes (denaturation), then chilled at 4°C and transferred to -20°C for storage.

3.2.7.2. Nodule gene targets

To gain an accurate insight in to the relative viability of *Wolbachia* and its host *O. ochengi* in the extirpated nodules, qRT-PCR was employed to quantify indicators of worm and bacteria vitality. Glutathione S-transferases (GSTs) are multifunctional proteins that have a role in the detoxification of cytotoxic products (Liebau et al., 2000). Using immunohistochemistry, in other studies *Ov*GST1 antisera has been used to demonstrate the rapid decline of *Ov*GST1 in *O. volvulus* within nodules extirpated from patients treated with doxycycline (Hoerauf et al., 2003b), illustrating that *Ov*GST1 is a reliable indicator of worm viability. In this study, *O. ochengi* GST1a (*Oo*GST1a) transcripts were quantified from the adult worms and used as an indicator for worm vitality, in comparison with 16S rRNA transcripts from

Wolbachia [previously validated using Aa23 cells *in vitro* (Makepeace et al., 2006)]. The ratio of cDNA copies per nodule provided a direct comparison of the *Wolbachia* and *O. ochengi* levels during the course of the experiment.

Transcripts of the bovine neutrophilic cytokines interleukin-8 (CXCL8) and the growth-regulated protein family [(GRO; CXCL1-3) see section 1.9.1.2], together with the eosinophilic cytokines eotaxin (CCL11) and interleukin-5 [(IL-5) see Section 1.9.2.1], were quantified alongside a "housekeeping" gene (bovine 28S rRNA) that was used for normalisation (Rosbottom et al., 2007).

3.2.7.3. Production of positive control cDNA for interleukin-8

Prior to analysis of nodule extracts, the performance of the qRT-PCR assay for IL-8 was evaluated with positive control cDNA obtained from bovine PBMC's. Bovine blood (20 ml) from an adult *Bos taurus* in the UK was collected via jugular venepuncture into lithium-heparin Vacutainers® (Becton Dickinson). Blood was diluted in an equal volume of RPMI medium containing 1% heparin, then overlaid onto Lymphoprep™ (Axis-Shield; 1:1 ratio to blood solution) and centrifuged for 45 minutes at 1,200 *g* (without brake). The PBMC layer was removed and diluted in 1% heparinised RPMI medium to a final volume of 45 ml. The suspension was centrifuged at 2,000 *g* for 10 minutes, the cell pellet re-suspended in 1% heparinised RPMI medium, and viability assessed using trypan blue exclusion. PBMCs were diluted to 0.9×10^7 cells ml^{-1} in RPMI medium (supplemented with 10% foetal calf serum and penicillin-streptomycin at 100 UI ml^{-1}, placed in 24-well cell culture plates and incubated with 5 $\mu g\ ml^{-1}$ concanavalin A (Sigma) at 37°C (5% CO_2) for 24 or 48 hours before harvesting. The cells were washed twice in 1% heparinised RPMI medium and quantified using a Neubauer counting chamber, washed in PBS, then

placed in 9 volumes of. RNA*later*® (for simultaneous RNA extraction, cells harvested at 24 hours post-stimulation were stored at 4°C). Cells were centrifuged (2,000 *g* for 10 minutes) and RNA was extracted from ~1 × 10^7 cells using the RNAqueous® Midi Kit (Ambion) as described 3.2.7.1. The RNA was quantified using a Nanodrop-1000 spectrophotometer and reverse-transcribed as detailed in Section 3.2.7.1.

3.2.7.4. Design of PCR primers and quantitative standards

Complete coding sequences for specific targets were sought from the NCBI database, prioritising fully published data where available. Sequences were transferred to the Primer3 v. 0.4.0 website (http://frodo.wi.mit.edu/primer3/), and the following parameters were selected: a melting temperature (T_m) within the range of 59 - 61°C with a maximum difference of ±1°C, a product size of approximately 100 base pairs, and no tracts containing >2 guanine or cytosine bases. All resultant sequences are documented in Table 3.3. As the bovine GRO protein family is encoded by three genes (Modi and Yoshimura, 1999), sequences were aligned using CLUSTALW (http://clustalw.genome.jp/) software to ensure that all three gene products were amplified. Alignments between human and bovine 28S rRNA sequences were also performed in order to select primer binding sites that were unlikely to amplify human DNA from the laboratory environment. Primers were obtained from Eurofins MWG Operon.

3.2.7.5. Construction of standard curves

Each assay included a specific standard curve for absolute quantification, using single-stranded oligonucleotides representing the full-length amplicons. These were obtained by custom synthesis from Sigma and diluted to a concentration of 10 μM.

To prevent low concentrations of DNA adhering to plasticware, $\log_{10}$ serial dilutions (5×10^{6} to 5×10^{-1} copies μl^{-1}) were performed in 100 μg ml^{-1} yeast tRNA (Invitrogen) and stored at 4°C.

target	orientation	primer sequence (5'→3')	amplicon size (bases)	amplicon T_m (°C)	sensitivity (copies reaction^{-1})	NCBI accession no.	species	reference
16S rRNA	sense	TTGCTATTAGATGAGCCTATATTAG	99	78	<50	AJ010276.1	*Wolbachia* (strain *w*Oo)	(Bandi et al., 1998)
	antisense	GTGTGGCTGACCATCCTCT						
GST1a	sense	CATGCTCATGACTTTGGATGA	85	77	<10	AF265556.1	*Onchocerca volvulus*	(Krause et al., 2001)
	antisense	GGGCATTTGTCCAATTTTGT						
28S rRNA	sense	TTTAAGCAGGAGGTTGTCAGG	77	78	<10	DQ222453.1	*Bos taurus*	Unpublished
	antisense	GCAACGTCCTTATGAACACTTG						
IL-8	sense	GTACAGAACTTCGATGCCAATG	94	77	<50	FJ223565.1	*B. indicus*	Unpublished
	antisense	TGTGGCCCACTCTCAATAACT						
IL-5	sense	TGGCAGAGACCTTGACACTG	94	78.5	<10	EU915048.1	*B. indicus*	(Freeman et al., 2008)
	antisense	TGGTGATTTGTATGCTGAGGA						
eotaxin	sense	GCCAGCTTCTATTCCAACCA	103	78	500	NM_205773.2	*B. taurus*	(Zimin et al., 2009)
	antisense	AGGACATTTGCTGCTCGTG						
GRO protein family	sense	CAAGAACATCCAGAGCGTGA	100	82, 84[a]	<10	NM_175700.1[b]	*B. taurus*	(Modi and Yoshimura, 1999)
	antisense	GTTGAGACACACTTCCTGACCA				NM_001048165.1[c]	*B. taurus*	
						NM_174299.2[d]	*B. taurus*	
						NM_001046513.1[d,e]	*B. taurus*	Unpublished

Table 3.3. Oligonucleotide primer sequences used for quantitative PCR

[a]Two of the samples assayed (4.2%), both from the melarsomine treatment group, produced two peaks in the melting curve analysis. This suggests that two members of the GRO protein family may have been expressed simultaneously in these rare cases.

[b]GRO-α (CXCL1)

[c]GRO-β (CXCL2)

[d]GRO-γ (CXCL3)

[e]Sense and antisense primers for the GRO protein family showed 100% homology with sequences NM_175700.1, NM_001048165.1 and NM_174299.2, but two mismatches each for sequence NM_001046513.1

3.2.7.6. Assay conditions

The second step of the qRT-PCR was performed using reagents supplied in the SensiMix Two-Step Kit (Quantace). Each reaction (final volume, 20 µl) comprised 200 nM each primer, 1× SYBR® Green I, and 1 µl DNA template in 1× SensiMix (dT). For each sample, two concentrations of template were assayed in single wells; no-template controls (NTC; ultrapure water replacing template) and standards were included in duplicate on each plate. Reactions were conducted on a MiniOpticon Real-Time PCR Detection System (Bio-Rad) under the following conditions: 10 minutes at 95°C (hot-start polymerase activation); then 35 cycles of 15 seconds at 95°C (denaturation), 30 seconds at 55°C (annealing), and 15 seconds at 72°C (extension); followed by a melting curve (55 - 95°C) to confirm product specificity. The NTC were negative in each reaction, confirming the absence of contamination. Copy numbers of targets quantified on each plate were interpolated by linear regression, and the value closest to the centre of the standard curve (or if inhibition was suspected, the value resulting from the greater dilution) was used for subsequent analysis. Copy numbers were normalised by subtraction of the corresponding RT- value, which in 96% of cases was negligible (<3% of the RT+ quantity).

3.3. Comparison of bovine granulocyte migration towards extracts of *Wolbachia*-replete or depleted *Onchocerca ochengi*.

3.3.1. Rationale and experimental design

Wolbachia-containing *O. volvulus* extracts stimulate the migration of human neutrophils *in-vitro* (Rubio de Kromer et al., 1998), in contrast to *Wolbachia*-depleted extracts (Brattig et al., 2001). Although *O. ochengi* were shown to induce the activation of human neutrophils *in-vitro* (Brattig et al., 2001), there have been no reports on the chemotactic activity of bovine granulocytes in this system. Therefore this experiment was designed to investigate whether *Wolbachia*-containing *O. ochengi* extracts exert a chemokinetic or chemotactic effect on bovine neutrophils; and reciprocally, whether *Wolbachia*-depleted (*O. ochengi*), or *Wolbachia*-free (*Setaria labiatopapillosa*) filarial extracts, exert a chemokinetic or chemotactic effect on bovine eosinophils.

3.3.2. Animal sourcing and experimental conditions

3.3.2.1. Ethical considerations

Experiments were authorised under Home Office Licence PPL 40/3243, and approved by the University of Liverpool's local ethical review process. Fresh blood collected via the jugular vein of slaughtered cattle was used for the optimisation of granulocyte purification. For all definitive experiments using *O. ochengi* extracts, a single 3-year-old Dexter bull was used as a donor to ensure minimal variation within blood parameters. The animal was kept at pasture at Ness Heath Farm, University of Liverpool Leahurst Campus. Regular faecal egg counts were performed to avoid unnecessary administration of anthelmintics.

3.3.3. Granulocyte Purification

3.3.3.1. Separation of bovine polymorphonuclear leukocytes

From a search of the literature, six detailed publications (Table 3.4) of the purification of granulocytes from ruminant species were reproduced within our laboratory, from which a definitive protocol was developed to coincide with the requirements for this experiment. Primary parameters to be considered were as follows.

Blood volume required: for each chemotaxis experiment, a minimum of 1 L of blood was required to yield sufficient granulocytes to perform all migration assays. This resulted in protocols using large volumes of anticoagulant, or the use of small collection tubes, inappropriate.

Equipment available (e.g. fixed rotors): some protocols required specific pieces of equipment which were unavailable within our laboratory. The results using dissimilar equipment were unreliable.

Table 3.4. Protocols for ruminant PMN separation. Steps indicated in blue font were adapted for use in the definitive protocol

Author(s)	Species	Anticoagulant	Spin time(s)	RBC lysis	Cell separation medium	Reported cell purity
(Lamote et al., 2007)	Bovine	Alsever's solution (1:1 blood)	300 *g* for 10 minutes	10 minutes in NH_4Cl lysis buffer	Percoll (specific gravity of 1.094 mg ml^{-1})	95% neutrophils
(Woldehiwet et al., 2003)	Ovine	EDTA (-coated Vacutainers)	400 *g* for 20 minutes	9 volumes sterile distilled water for 20 s followed by 1 volume of 9% (w/v) NaCl.	Percoll gradient 55% - 70%	94% neutrophils at 65% Percoll, 23% eosinophils at 55% Percoll
(Chambers et al., 1983; Chambers et al., 1985)	Bovine	0.11 M Sodium citrate (1:1 blood)	400 *g* for 15 minutes	0.83% NH_4Cl for 5 minutes followed by an equal volume of citrate saline solution	Percoll gradient [49% - 74% (Cambers et al., 1983); 68% Percoll (Cambers et al., 1985)]	>95% PMN (paper 172); >60% eosinophils (Cambers et al., 1985)
(Roth and Kaeberle, 1981)	Bovine	Acid citrate dextrose solution (2x concentration of standard formula A)	1,000 *g* for 20 minutes	2 volumes phosphate-buffered distilled water for 50 seconds followed by 2.7% NaCl	Ficoll-Hypaque (specific gravity of 1.135)	>90% eosinophils (band) and neutrophils (pellet)
(Freiburghaus and Jorg, 1990)	Bovine	2.5% Na_2-EDTA	2,300 *g* for 20 minutes, followed by 2,300 *g* for 30 minutes	Two volumes of distilled water for 60 seconds followed by 4.5% NaCl solution	68% Percoll solution	92% eosinophils
(Riding and Willadsen, 1981)	Bovine	0.1 M Na_2.EDTA	900 *g* for 15 minutes	Equal volume sterile distilled water, then 0.46 M NaCl	Percoll gradient (1.090 g ml^{-1} stock), then centrifuged (fixed-angle rotor @ 20,000 *g*, 20 mins)	92% eosinophils at 1.096 - 1.111 g ml^{-1} density; 95% neutrophils at 1.106 - 1.120 g ml^{-1} density

3.3.3.2. Definitive Protocol

Twenty ml of 10% sodium-EDTA [Sigma (Soldan, 1999)] was used to coat 1 L veterinary dry sterile blood bags (Kruuse). The bull was lightly sedated with 1 ml xylazine (Rompun®, Bayer Animal Health) injected into the gluteal muscle, then restrained using a crush and head collar. The mid-neck was clipped of hair and aseptically prepared using chlorhexidine. An intradermal bleb of local anaesthetic [Willcain (Arnolds)] was instilled and the skin incised using a sterile scalpel. A 12 gauge needle was attached to the giving set and anticoagulant was flushed retrograde. Jugular occlusion was carried out via digital pressure for one minute before venepuncture. One litre of blood was collected whilst gently rocking the collection bag to avoid coagulation. After blood collection, the needle was removed and digital pressure applied to the neck to avoid the formation of a haematoma. The blood bag was sealed with haemostats and transported to the laboratory on ice.

Working in the laminar flow cabinet to maintain asepsis, the blood was decanted into 50 ml centrifuge tubes, and centrifuged for 20 minutes at 2,300 *g*, 4° C. Ten millilitres of packed cells was removed from the area immediately below the buffy coats, and transferred to four new tubes (200 ml in total). These tubes were centrifuged at 2,300 *g*, 4°C for 30 minutes. Any wrinkled film that had appeared was removed together with the top 10 ml of cells, and collected in to a sterile 1 L glass beaker (40 ml total) (Freiburghaus and Jorg, 1990). Red blood cells were lysed in 400 ml ammonium chloride (4°C) solution (appendix 12.8.3) for 10 minutes, then centrifuged at 400 *g*, 4°C for 10 minutes (Lamote et al., 2007). The supernatant was removed, and the remaining cell pellets were washed twice in PBS containing 1.2 mM EDTA (Biowhittaker ®) and centrifuged at 200 *g*, 10 minutes, 4°C. The pellets were pooled and reconstituted in 5 ml PBS-EDTA, layered onto a Percoll® (Sigma)

gradient (see 3.3.3.3), and centrifuged at 400 *g* for 15 minutes at 4°C (without brake) (Chambers et al., 1983). Cell bands were collected and washed twice in PBS-EDTA as before, then re-suspended in PBS-EDTA. Total viable cell counts were performed via trypan blue-exclusion using a Neubauer counting chamber. Thin smears of each fraction were stained using a Romanowsky stain [Quick-Diff® (reastain) (Woldehiwet et al., 2003)] and differential cell counts were performed.

3.3.3.3. The Percoll® gradient

Percoll® stock solution (appendix 12.8.3) was diluted in PBS (Sigma) to attain the concentrations in Table 3.5. A discontinuous density gradient of decreasing concentrations was constructed by passing each dilution through a 25 gauge hypodermic needle bent at 90 degrees (bevel pointing upwards), placed just below the surface of the preceding layer. The gradient was calibrated using a Percoll® density marker bead kit (GE Healthcare Life Sciences) according to the manufacturer's instructions. Briefly, the beads were equilibrated in 1 ml distilled water overnight, and 0.1% w/v thimerosal was added to each vial to prevent bacterial growth. Fifteen microlitres of each bead suspension were added to 0.11 M sodium citrate buffer (Appendix 12.8.3) to a final volume of 2.5 ml and placed on top of a Percoll® gradient constructed in a 15 ml centrifuge tube, using 2.5 ml of each concentration detailed in Table 3.5. The gradient was centrifuged at 400 *g* for 15 minutes (without brake) and each coloured band separated out within the corresponding part of the gradient as shown in table Table 3.5.

Table 3.5. Verification of discontinuous Percoll® gradient used for the separation of bovine eosinophils and neutrophils.

Band colour	Density (g ml^{-1})	Distance from meniscus to band (mm)	Percoll density equivalent (%)	
Red	1.064	20	49	
Blue	1.074	42	55	
Yellow	1.088	63	62	
Green	1.102	100 (pellet)	68	Eosinophil band (1.092-1.111 g ml^{-1})
Red	1.120	100 (pellet)	74	Neutrophil pellet (1.106-1.120 g ml^{-1})

3.3.4. Cell migration Assays

3.3.4.1. Preparation of Chemoattractants

3.3.4.1.1. Controls

The yeast cell wall component zymosan, a bovine neutrophil (Shuster et al., 1997) and eosinophil (Vogel et al., 2005) chemoattractant, activates the complement cascade after incubation with serum, via the activation of C5a and its derivative $C5a^{desArg}$ (Fischer and Czarnetzki, 1982; Rainard et al., 1998). Zymosan-activated serum (ZAS) is an effective positive control at a concentration of 1 mg ml^{-1} for *in-vitro* bovine PMN migration assays (Vogel et al., 2005; Yu et al., 2010). Thus, ZAS was prepared according to a protocol by Junger et al (1993). Briefly, 20 ml newborn calf serum was warmed to 27°C in a water bath, and then incubated with 100 mg zymosan A (Sigma) at 37°C on a shaking platform for 30 minutes. The serum was centrifuged (1,000 *g* for 20 minutes), then removed from the zymosan pellet and

incubated at 56°C in a water bath for 30 minutes. The ZAS was stored in aliquots at -80°C.

Recombinant bovine IL-8 [(*rb*IL-8) see section 1.9.1.2], a chemokine shown to positively attract bovine neutrophils but not eosinophils *in-vitro* (Caswell et al., 1999), was purchased from Kingfisher Biotech, Inc (St Paul, Minnesota, USA). Lyophilised solid was dissolved in 1% bovine serum albumin (BSA) to a final concentration of 1 μg ml^{-1}, then stored at -80°C.

Bovine eotaxin (see Section 1.9.2.1), an effective eosinophil chemoattractant *in-vitro* (Vogel et al., 2005), was not commercially available. However, as the chemokine recombinant murine eotaxin (*rm*eotaxin) was found to attract ovine eosinophils *in-vitro*, (Wildblood et al., 2005), cross-reactivity within the Bovidae family was deemed probable. Thus, *rm*eotaxin (Peprotech) was reconstituted in PBS supplemented with 0.1% heat-treated newborn calf serum to a final concentration of 1.6 μg ml^{-1}, and stored at -80°C.

The reported chemotactic activity of the bacterial cell wall component PDG on granulocytes is ambiguous. In human studies, PDG was found to be a specific activator for neutrophils (Mattsson et al., 2003), and exerted a chemotactic effect on neutrophils in normal human serum via activation of the alternative complement pathway (Riber et al., 1990). Conversely, another study reported the suppression of neutrophil chemotaxis in the presence of *Staphylococcus aureus* PDG, suggesting a bacterial defence mechanism (Musher et al., 1981). However, since PDG monomers (muramyl dipeptides) are synthesised by *Wolbachia*. (Foster et al., 2005), and as these were thought to stimulate an inflammatory response via TLR-2 (Bougarn et al., 2010), PDG constituted a candidate positive control for *in-vitro* neutrophil

chemotaxis. Peptidoglycan from *Bacillus subtilis* (Fluka) was suspended in PBS to a stock concentration of 20 mg ml^{-1}, ultrasonicated in a PUL55 ultrasonic bath (Kerry Ultrasonics, Hitchin, England) at approximately 30 kHz for three 10-second bursts, and stored at -80°C.

Ascaris suum, an intestinal roundworm of pigs, and *Fasciola hepatica*, a liver fluke which parasitizes sheep and cattle, attract bovine eosinophils *in-vivo* (Duffus et al., 1980; Grewal and Babiuk, 1979) and guinea pig eosinophils *in vitro* (Horii et al., 1988). Fresh *A. suum* were collected from pig faeces and *F. hepatica* from sheep livers at a local abattoir, and frozen at -80°C. The helminths were cut into approximately 1 mm^3 pieces on ice, then homogenised in PBS for three-30-second bursts, using a Polytron® PT1200 homogeniser equipped with a PT-DA 1205, 2.5-mm diameter dispersing element (Kinematica, Lucerne, Switzerland). The homogenates were centrifuged at 21,900 *g* (4°C) for 2 hours, and the supernatants were subjected to the Bradford assay (Section 3.3.4.1.4) to quantify protein prior to storage at -80°C.

Adult *S. labiatopapillosa,* a *Wolbachia*–free filarial parasite of cattle (Casiraghi et al., 2004), were removed from the peritoneal cavities of cattle slaughtered at an abattoir in Ngaoundéré, Northern Cameroon. Non-gravid females were placed in PBS, stored and transported to Liverpool at -80°C, and subsequently prepared for use in cell migration assays as detailed in Section 3.3.4.1.2.

3.3.4.1.2. Wolbachia-depleted Onchocerca ochengi.

Wolbachia-depleted *O. ochengi* were harvested from a group of animals that had received a short treatment regimen of oxytetracycline (Terramycin® LA, Pfizer) at IRAD, Ngaoundéré, Northern Cameroon. Fifteen naturally and heavily infected

Gudali breed cattle received fourteen daily doses of Terramycin LA® (Pfizer) at10 mg kg^{-1} as detailed in Appendix 12.2. A significant reduction of *Wolbachia* from post-treatment worms was confirmed by comparative immunohistochemical staining of nodule sections [using the DakoCytomation LSAB2 System kit (see Section 3.2.5)] removed at four and eight weeks post-treatment. Blinded screening of nodule sections revealed a general reduction in *Wolbachia* in the eight-week post-treatment worms as determined by reactivity of an anti-WSP polyclonal antibody [as seen in Figure 8.2.2 *(c)*]. Nodules were harvested from the hides of cattle slaughtered 10 - 12 weeks post-treatment, and the filariae were sexed and separated (females were further divided into gravid and non-gravid groups) using a dissection microscope. Filariae were stored and transported at -80°C in PBS.

3.3.4.1.3. Preparation of worm homogenates for chemotaxis experiments

Worm samples were removed from storage, transferred into a sterile tray placed on ice, and finely chopped using a scalpel. The worm fragments were suspended in 1 ml endotoxin-free PBS, combined with 1 g of beads (1:1 blend of 1 mm glass and 0.1 mm zirconia-silica) and homogenised in a Mini-Beadbeater™ (Biospec Products) for four 1-minute cycles at 4,800 rpm, with cooling on ice for 2 minutes between each cycle. The homogenates were transferred to microcentrifuge tubes, and centrifuged at 17,000 *g* at 4°C for two hours. The protein content of the soluble supernatant was measured using the Bradford assay as described in section 3.3.4.1.4, and the extracts were stored at - 80°C. The insoluble pellets were also stored at -80°C prior to Western blotting (Section 3.3.4.2). For use in chemotaxis assays, worm homogenates were diluted to the desired concentration in enriched Dulbecco's modified Eagle's medium [DMEM (Appendix 12.8.3)].

3.3.4.1.4. Bradford protein assay

The protein concentrations of supernatants from parasite homogenates were quantified using a Dynex Technologies™ microplate reader equipped with Revelation™ version 3.2 software. Samples and BSA standards (Sigma) were incubated with the Bradford reagent (Sigma) in flat-bottomed 96-well microplates according to the manufacturer's instructions, and optical densities were obtained at 570 nanometres. Protein concentrations were interpolated using linear regression against a standard curve (serial dilutions of BSA from 0.19 - 1.5 mg ml^{-1}). The concentrations of each parasite extract are displayed in table Table 3.6.

Table 3.6. Protein content of parasite somatic supernatant as determined by Bradford assay.

Worm homogenate	Protein content (mg ml^{-1})
Ascaris suum	0.39
Fasciola hepatica	15.0
Setaria labiatopapillosa	0.68
O. ochengi male (*Wolbachia*-positive)	1.0
O. ochengi male (*Wolbachia*-negative)	1.3
O. ochengi female (non-gravid, *Wolbachia*-positive)	5.6
O. ochengi female (non-gravid, *Wolbachia*-negative)	9.7

3.3.4.2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) and Western blotting

To determine that the *O. ochengi* worms obtained from the antibiotic-treated cattle were successfully depleted of *Wolbachia* in comparison to the control animals, and to semi-quantify the WSP content of the worm homogenates, SDS-PAGE followed by Western blotting was performed on the *O. ochengi* insoluble pellets which remained after homogenisation (Section 3.3.4.1.3). Polyacrylamide gels were prepared in-house (Appendix 12.8.3). Protein extracts from the naturally *Wolbachia*-free nematode *S. labiatopapillosa,* and recombinant WSP, were used as negative and positive controls, respectively. Briefly, two 12.5% resolving gels were poured between 1-mm spacer plates (Bio-Rad), levelled with water-saturated butan-2-ol, and then overlaid with a 5% stacking gel into which a 1mm comb was placed to produce wells. The proteins were denatured by boiling the pellets for 5 minutes in 1× Laemmli sample buffer (Appendix 12.8.3) at 100°C in a heating block. Each gel was loaded with 0.5 µl Dual Color Precision Plus Protein™ Standards (Bio-Rad), followed by 100 µg sample protein per well (for gels destined for electroblotting), or

10 µg per well [for gels destined for staining with colloidal Coomassie Brilliant Blue (Appendix 12.8.3)]. The proteins were separated by size during electrophoresis (70 volts for stacking, then 200 volts for resolving) within a tank (Bio-Rad Mini Protean II™) containing 1 × TGS buffer (see Appendix 12.8.3 for 10 × stock buffer) until the dye front reached the bottom of the gel. For Western blotting, the gels were placed in cassettes (Bio-Rad Mini Cell transfer apparatus) immersed in a tank (Bio-Rad Mini Protean II™) of transfer buffer (Appendix 12.8.3), and electroblotted (20 volts overnight, in a refrigerated unit) onto a nitrocellulose membrane (Hybond, Amersham Biosciences). The membrane was rinsed with distilled water and incubated in blocking buffer (Appendix 12.8.3) for 3 hours, then with anti-WSP antibody or normal rabbit serum diluted 1/400 in blocking buffer for 2 hours. Finally, the blot was incubated with goat anti-rabbit IgG-alkaline phosphatase secondary antibody [(Sigma), diluted 1/30,000 in blocking buffer (Appendix 12.8.3)] for 45 minutes on a rocking platform. The nitrocellulose was washed three times (5 minutes) in fresh transfer buffer between each step. The blots were developed for 70 seconds in 1-Step™ nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3`-indolyphosphate p-toluidine salt solution (Thermo Scientific), diluted 1:1 in water, and the reaction terminated using distilled water.

3.3.4.2.1. Protein quantification of filarial worm pellets

The Protein dotMETRIC™ assay (G-Biosciences®) kit was used to quantify the small insoluble pellets remaining after filarial homogenisation. The accuracy of the assay was verified using a known concentration of BSA prior to use. The filarial pellets were boiled (100°C for 5 minutes) in an equal volume of 1× Laemmli sample buffer (Appendix 12.8.3) using a heating block, and then centrifuged at 17,000 *g* for 10 minutes. One microlitre of protein solution was added to the supplied dilution buffer

to make a final volume of 20 µl and spotted onto test strips using a capillary tube. The strips were developed according to the manufacturer's instructions (Protocol 2), and the diameter of the protein spots was measured using the supplied Protein dotMETRIC™ scale. The protein concentration was calculated from the mean of four replicates (Table 3.7).

Table 3.7. Protein concentration of filarial worm pellets as determined by the Protein dotMETRIC™ assay.

Worm pellet	**Mean protein concentration (mg ml^{-1})**
Setaria labiatopapillosa	9.7
O. ochengi male (*Wolbachia*-positive)	6.8
O. ochengi male (*Wolbachia*-negative)	8.0
O. ochengi female (non-gravid, *Wolbachia* positive)	9.9
O. ochengi female (non-gravid, *Wolbachia* negative)	9.7

3.3.4.3. Chemotaxis-chemokinesis assays.

Preliminary experiments involved a series of trial assays in order to optimise the cell migration parameters, comprising:

- Assay plate manufacturers (Nunc, Becton Dickinson, Millipore);
- Mock chemoattractant diffusion assays;
- Cell migration assay incubation period (1 or 2 hours);
- Assay plate pore size (3 or 8 µm);
- Positive control stimuli (PDG, *rm*eotaxin, *rb*IL-8, ZAS, *A. suum*, *F. hepatica*).

Assay plates were chosen according to their membrane materials and working volume parameters. To determine the optimal assay length within our laboratory protocol we performed a simple diffusion test to ascertain the steepness of the gradient followed by a mini cell migration assay to determine the effect of incubation time on total cell migration. Trypan blue was used as a marker in the lower wells. The plates were incubated at 37°C and the diffusion of trypan blue into the upper chamber was assessed by eye every 15 minutes until the first plate reached equilibrium. For the mini cell migration assay, counts of intact cells that were adhered to the bottom of the membranes after incubation with chemoattractants for either one or two hours were compared. For the pore size experiments, cells were incubated in transwell systems containing pores of either 3 μm or 8 μm (Nunc™). Neutrophils and eosinophils were quantified after incubation at 37°C for 2 hours. To ascertain reliable positive controls, cell migration assays were performed using chemoattractants that were found to be effective in published studies (Section 3.3.4.1.1). Cell migration numbers for positive controls were divided by baseline migration numbers in the negative control (DMEM) chambers, to reveal cell migration indices. Controls with the highest migration indices were used in the definitive experiments.

For the definitive cell migration experiments, bovine granulocytes were separated using the Percoll discontinuous gradient (Section 3.3.3.3), and cell fractions were re-mixed in enriched DMEM (Appendix 12.8.3) to produce an overall cell concentration of 5×10^6 ml^{-1} cells with an eosinophil content of 50%. Chemoattractants (200 μl per well; six replicates) were prepared and warmed in a 37°C incubator within Multiscreen MIC 96-well companion plates (Millipore™). The filter plate was seated into the companion plate, and a 50 μl aliquot of cell

suspension was placed into each upper chamber prior to incubation at 37°C (5% CO_2) for one hour. The filter plate was washed with PBS, air-dried quickly, stained with Quick-Diff (Reastain), and dried overnight. The filters were removed using a scalpel and mounted (underside facing uppermost) onto glass slides using gridded cover-slips (Electron Microscopy Sciences, Pennsylvania, USA) and a water-soluble mountant (VectaMount™ AQ, Vector Laboratories). Cells that had passed through the filter (*i.e.*, those in the uppermost field of view) were visually differentiated and quantified microscopically at 400× final magnification in ten randomly-selected (www.randomizer.org) grid references.

4. *In-vitro* studies on the effects of melarsomine on *Wolbachia*

4.1. Introduction

The organic arsenical compound melarsomine was assessed for its suitability for use as a non-antibiotic control against *O. ochengi in-vivo*. In a previous study, melarsomine was completely macrofilaricidal in cattle within 5 months of treatment; as evidenced by an 86% decrease in nodule load, with only small, hard nodules containing only dead worms remaining (Tchakouté et al., 2006). The effects of melarsomine on prokaryotic cells were unknown but in contrast with Tchakouté et al and in the same model, nodule resolution and worm death were recorded 9 months after treatment using the antibiotic oxytetracycline, and followed *Wolbachia* depletion (Gilbert et al., 2005; Langworthy et al., 2000). Thus, a difference in the mechanism of worm killing between the two drugs was inferred, in which the relatively fast macrofilaricidal activity of melarsomine was deemed to result from a direct toxic effect. However, to confirm this using a reliable and convenient *in-vitro* model, an experiment using the Aa23 mosquito cell line (which is naturally infected with *Wolbachia*) was performed. Cells were treated with melarsomine hydrochloride, doxycycline hyclate [a tetracycline known to deplete *Wolbachia* in this system (Hermans et al., 2001; Makepeace et al., 2006)], or sterile water for 3 or 7 days preceding quantification of *Wolbachia* (16S) and Aa23 host cell (18S) rRNA using qRT-PCR (Section 3.1).

4.2. Results

4.2.1. Effect of treatment on *Aedes albopictus* host cells

At 3 days; 18S rRNA copy numbers in melarsomine-treated cells were lower than in doxycycline treated cells [Figure 4.2.1 *(a)*; mean difference ~0.5 log], but this was not significant. At 7 days, the difference was greater due to the inhibitory effect of melarsomine causing a mean difference of -1.5 log (p = 0.008; GLM followed by Dunnett's post hoc t-test) compared to control data, while doxycycline did not cause any significant difference in growth. The difference in 18S rRNA values was highly significant between drugs [$p < 0.001$, (Appendix 12.7)], but equivalent between the three concentrations tested.

4.2.2. Effect of treatment on *Wolbachia*

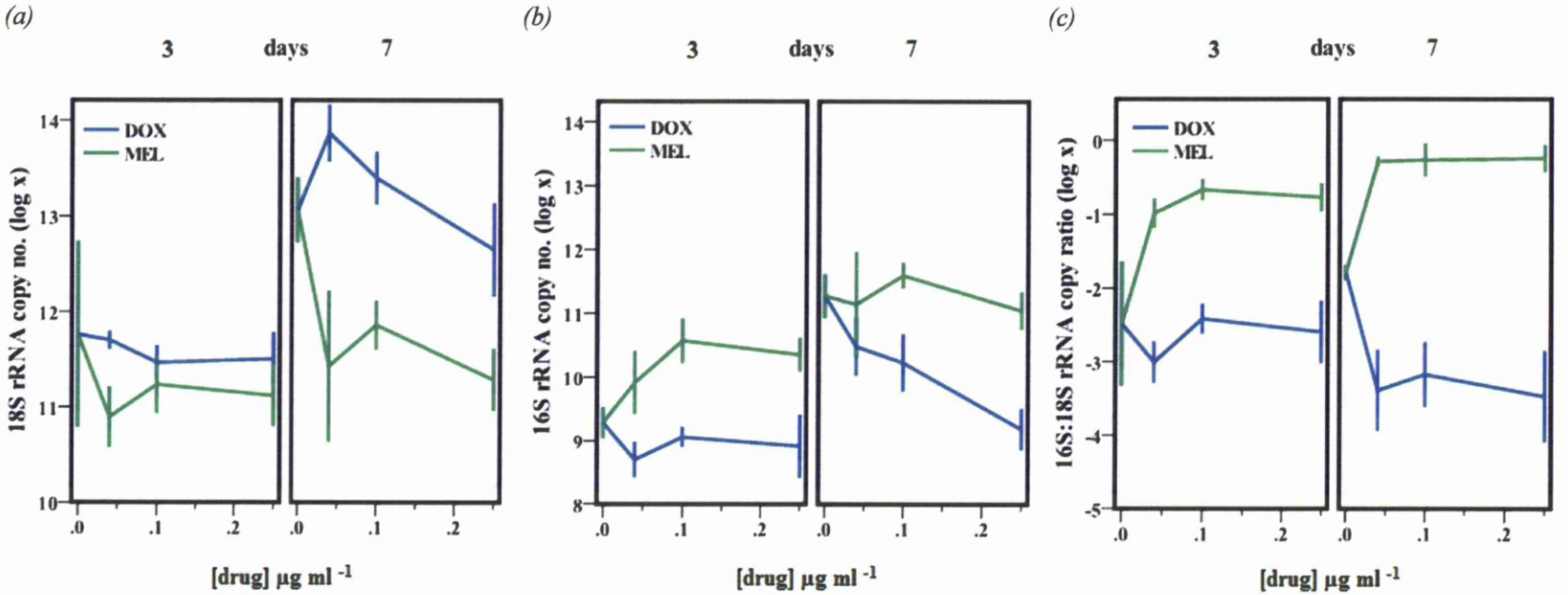
At 3 days, there was a significant difference between 16S rRNA copy numbers in treated cells [p <0.001, (Appendix 12.7)], with 16S rRNA from melarsomine-treated cells increased by an average of ~1.0 log [Figure 4.2.1 *(b)*; Dunnett's post-hoc t-test, $p = 0.023$] compared to control data. Melarsomine continued to be ineffective after 7 days of treatment; conversely, doxycycline reduced 16S rRNA to a mean of 1.3 log below the control level by day 7 (Dunnett's post-hoc t-test, $p = 0.024$).

4.2.3. Analysis of the *Wolbachia*:*Aedes* rRNA copy number ratio

Figure 4.2.1 *(c)* shows that at 3 and 7 days, the 16S:18S rRNA ratios were highly significantly different with respect to the drug used (p <0.001 in each case, Appendix 12.7), with melarsomine causing a significant increase to the ratio at both time points [mean increase of 1.6 log and 1.7 log, respectively; $p = 0.002$ (Dunnett's post-hoc t-test)]. Conversely, the 16S:18S rRNA ratios were decreased after doxycycline

treatment at 7 days by a mean of 1.5 log [p = 0.002 (Dunnett's post-hoc t-test)] compared to control data.

Figure 4.2.1. Effect of melarsomine and doxycycline on Aa23 mosquito host cells and *Wolbachia* after a 3- or 7-day treatment



Quantification of *(a)* Aa23 mosquito host cell 18S rRNA, *(b) Wolbachia* 16S rRNA, and *(c)* 16S:18S rRNA ratio of copy numbers in response to treatment with doxycycline or melarsomine. Cultures were assayed in quadruplicate and data are presented on a logarithmic scale. Lines represent mean ± 1SE.

4.3. Discussion

The results from this experiment were a prerequisite for the use of melarsomine as a substance that would cause filarial death without exerting a specific effect on *Wolbachia*, thus confirming its suitability as a non-antibiotic control *in vivo*. Because there is a degree of natural fluctuation in the relative numbers of mosquito cells and *Wolbachia* during culture, the *Wolbachia*:Aa23 rRNA ratio provided the most informative insight into the net effects of the drugs. Melarsomine significantly increased the ratio at 3 and 7 days [Figure 4.2.1 *(c)*], an effect resulting from the continued growth of *Wolbachia* during melarsomine treatment, coupled with inhibitory effects on the host mosquito cells. Therefore, unlike doxycycline, melarsomine has no bactericidal or bacteriostatic activity against *Wolbachia*.

4.3.1. Arsenicals

The mechanism of action of melarsomine remains largely speculative, but the *in-vitro* results presented here are supported by the observation that melarsomine does not clear *Wolbachia* from worms within the lungs of *D. immitis*-infected dogs treated with melarsomine alone, as revealed by anti-WSP staining, compared to no such staining in lungs from dogs treated with regimens including doxycycline (Kramer et al., 2008). If melarsomine had an anti-*Wolbachia* effect in heartworm infection, a decrease in anti-WSP staining in the lung parenchyma from the melarsomine-treated dogs would be expected.

In *Litomosoides carinii*, arsenical compounds were found to be macrofilaricidal (Dickerson and Thompson, 1966) via the inhibition of glutathione reductase (Bhargava et al., 1983), which was found to involve a two-stage process in *Setaria*

digitata and *O. gutturosa* (Muller et al., 1995). Whilst adenosine triphosphate (ATP) metabolism is reportedly unaffected (Bhargava et al., 1983), arsenicals are thought to inhibit the uptake and metabolism of glucose, and also alter the structure of the intestinal epithelium within the worm (Subrahmanyam 1987). In the protozoan *Trypanosoma brucei*, melarsomine may diminish ATP by the inhibition of glycerol-3-phosphate, eventually leading to reduction of motility and cell lysis (Denise et al., 1999).

The use of arsenic and its compounds portray a rich history. For centuries arsenic was used in the treatment of cutaneous disease; Hippocrates apparently prescribed the topical use of orpiment, a natural form of arsenic for the treatment of abscesses; then centuries later Galen recommended arsenic for the treatment of leprosy and tuberculosis (reviewed by Riethmiller, 2005). Salvarsan, the first man-made antibiotic, was synthesized from atoxyl by Ehrlich and Bertheim in the early 20th century, and was used to treat *Treponema pallidum*, the aetiological agent of syphilis, until it was superseded by penicillin in the 1940s. The efficacy of atoxyl against *Trypanosoma brucei gambiense* was reported (Thomas and Mcgill, 1905), and the related compounds melarsoprol and melarsomine (brand name Immiticide®) are currently used in the treatment of African trypanosomiasis and canine heartworm, respectively. The high incidence of toxicity is a serious concern, however, and limits its use in humans (Wang, 1995). In light of the recognised efficacy of arsenical compounds against certain bacteria, the *in vitro* evaluation of melarsomine against *Wolbachia* was an important validation step before proceeding to the *in vivo* experiment.

4.3.2. Tetracyclines

The *Wolbachia*:Aa23 rRNA ratio [Figure 4.2.1 *(c)*] was significantly reduced in the doxycycline-treated cultures, which was a result of the decreased *Wolbachia* numbers compared to unaffected mosquito cells. This was an expected result from the positive control, as the anti-*Wolbachia* activity of lipophilic doxycycline had been established previously in the Aa23 cell line (Fenollar et al., 2003; Hermans et al., 2001; Makepeace et al., 2006). In this model, doxycycline shows superior effects in comparison to the less lipophilic oxytetracycline, probably due to the higher intracellular concentration attained via an increased cellular uptake of the drug (Hermans et al., 2001).

Tetracycline antibiotics are bacteriostatic via the inhibition of protein synthesis. However, in one study, doxycycline exerted a strong killing effect on *Wolbachia* within the mosquito Aa23 cell line, causing a ~6 log reduction in bacterial rRNA (as evidenced by qRT-PCR) after three weeks (Makepeace et al., 2006), which may indicate unique bactericidal activity against *Wolbachia.*

Initial *in-vivo* studies reported the degeneration of *Wolbachia* within female *B. pahangi* reproductive tracts (using electron microscopy), and the depletion of *Wolbachia* in *D. immitis* (using PCR), after a 30 - 35-day course of tetracycline given to infected jirds or dogs, respectively (Bandi et al., 1999). Similar results were reported after the *in-vivo* treatment of *L. sigmodontis* in mice with tetracycline hydrochloride (Hoerauf et al., 1999) and *O. ochengi* in cattle with oxytetracycline (Langworthy et al., 2000); both exerted a deleterious effect on *Wolbachia* as evidenced by electron microscopy (Hoerauf et al., 1999; Langworthy et al., 2000) and immunohistochemistry (Hoerauf et al., 1999).

The anti-*Wolbachia* effects of doxycycline have been established in numerous *in-vivo* studies including *W. bancrofti* in humans (Debrah et al., 2007; Hoerauf et al., 2003a; Taylor et al., 2005d), *O. volvulus* in humans (Hoerauf et al., 2001; Hoerauf et al., 2003b; Hoerauf et al., 2000a), and *D. immitis* in dogs (Bazzocchi et al., 2008). In one *in-vitro* study using *O. gutturosa* adult worms, doxycycline was also reported to exert macrofilaricidal properties (Townson et al., 2006); however the concentration of doxycycline used (5×10^{-5} M) exceeded the physiologically relevant minimum bactericidal concentration established against *Wolbachia* in the Aa23 mosquito cell-line (Hermans et al., 2001) by 100-fold, and thus aberrant direct effects against *O. gutturosa* cannot be ruled out.

Although doxycycline was the tetracycline of choice for the *in-vitro* experiment, the lack of availability of a parenteral formulation for large animals, coupled with the reports of toxicity in cattle in its currently available oral formulation (Chiers et al., 2004; Yeruham et al., 2002) rendered doxycycline inappropriate for use in the *in-vivo* experiment. Furthermore, the antifilarial properties of doxycycline have not yet been elucidated in the *O. ochengi* model; however, the macrofilaricidal effects of other tetracyclines are well-documented, and as such oxytetracycline is used as a "gold standard" anti-*Wolbachia* drug against which other drugs are compared when quantifying *Wolbachia* depletion in this model.

5. The effects of *Wolbachia*-replete and *Wolbachia*-depleted *Onchocerca ochengi* on the migration of bovine granulocytes *in-vitro*

5.1. Introduction

In-vitro cell migration assays were performed using purified bovine neutrophils and eosinophils to elucidate the chemotactic properties of *Wolbachia*-containing extracts of *O. ochengi*. To compare the migration of granulocyte populations to filariae without *Wolbachia*, assays included *O. ochengi* previously depleted of *Wolbachia* via oxytetracycline chemotherapy (verified by Western blotting), and the naturally *Wolbachia*-free species, *S. labiatopapillosa*.

A striking similarity between the sessile, nodule-forming *O. volvulus* and *O.ochengi* is the accumulation of neutrophils around the periphery of filariae throughout their life (Brattig et al., 2001; Nfon et al., 2006). Previous *in-vitro* cell migration assays demonstrated that neutrophil activation and chemotaxis was a result of the presence of *Wolbachia*, since the chemotactic response of neutrophils was reduced by 50 - 70% in the presence of *Wolbachia*-depleted female *O. volvulus* compared to untreated worm extracts (Brattig et al., 2001). In addition, WSP-derived from *Dirofilaria immitis* exerted a chemokinetic effect on canine neutrophils (Bazzocchi et al., 2003). In Brattig's study, *O. ochengi* extracts from untreated cows stimulated IL-8 production from human monocytes and mononuclear cells (Brattig et al., 2001); but cell migration data was not collected, and this remains the only published *in-vitro* bovine cell stimulation data with respect to *O. ochengi*.

5.2. Results

5.2.1. Initial assay parameters

Using a Percoll® discontinuous density gradient (Section 3.3.3.3), it was possible to separate bovine granulocyte populations into eosinophil-rich and neutrophil-rich fractions (Figure 5.2.1). Eosinophil-rich fractions were either evident at the 1.092 g ml^{-1} band or the 1.100 g ml^{-1} band, whereas the neutrophil-rich fraction consistently pelleted (Figure 5.2.1). Next, a preliminary chemotaxis experiment using polycarbonate cell culture inserts (Nunc™) established that both bovine neutrophils [Figure 5.2.2 *(a)*], and eosinophils [Figure 5.2.2 *(b)*] required 8 micron pores for quantifiable chemotaxis.

A preliminary diffusion assay (Figure 5.2.3), followed by cell migration assays (Figure 5.2.4), enabled the establishment of the optimal incubation time. Testing two systems, it was found that diffusion of trypan blue occurred more rapidly through Millipore™ cell culture inserts, which reached a maximum diffusion score of 2 at 45 minutes [Figure 5.2.3] before reaching a plateau. In comparison, diffusion was slower through Becton Dickinson™ cell culture inserts [Figure 5.2.3], reaching only partial equilibrium at 45 minutes before the plateau phase commenced. Complete equilibrium between chambers occurred after overnight incubation (data not shown). Cell migration assays (Figure 5.2.4) showed that neutrophil [Figure 5.2.4 ***(a)***] and eosinophil [Figure 5.2.4 ***(b)***] migration occurred after 1 hour in numbers sufficient for quantification.

Cell migration assays were then performed to establish reliable positive controls for neutrophils and eosinophils. Ten percent zymosan-activated serum (ZAS) was found to be a consistent chemoattractant for bovine eosinophils [Figure 5.2.5 *(b)*; Figure

5.2.6 *(b)*; Figure 5.2.7]. *Ascaris suum* extract was also chemoattractive for eosinophils (Figure 5.2.6), but both recombinant murine eotaxin [Figure 5.2.6 *(b)*; Figure 5.2.7] and *Fasciola hepatica* extract exhibited low potency or inconsistent effects [Figure 5.2.6 *(b)*]. In assays for reliable neutrophil chemoattractants, peptidoglycan was found to be ineffective. However, recombinant bovine IL-8 showed a similar chemotactic and chemokinetic migration index to ZAS [Figure 5.2.5 *(a)*], and was selected as a reliable neutrophil chemoattractant.

Western blot assays were performed to confirm the depletion of *Wolbachia* in *O. ochengi* removed from antibiotic-treated cattle. These clearly showed a single band in both of the *Wolbachia*-positive worm extracts, which aligned with recombinant WSP and the 25 kDa high molecular weight marker band (Figure 5.2.8). This contrasted with the absence of a 25 kDa band in both of the *Wolbachia*-depleted *O. ochengi* extracts, as was also the case for the *Wolbachia*-negative species, *Setaria labiatopapillosa* (Figure 5.2.8).

5.2.2. Cell migration assays using filarial extracts

Following the refinement of the cell purification and migration assay techniques, three definitive experiments were performed using filarial extracts (figures **5.2.9**; **5.2.10**; **5.2.11**). The positive controls selected for bovine neutrophils and eosinophils produced broadly similar effects throughout these experiments. ZAS preferentially attracted eosinophils across all three experiments (p <0.001), and was significantly more chemotactic than chemokinetic in two experiments (Table 5.1). In contrast, while *A. suum* was also highly chemoattractive for eosinophils (p <0.001) on all three occasions, it was significantly more chemokinetic than chemotactic in two experiments (Table 5.1). Recombinant bovine IL-8 (rbIL-8) was significantly

chemoattractive for neutrophils in two of the three experiments. The naturally *Wolbachia*-free *S. labiatopapillosa* was significantly more chemotactic than chemokinetic across both cell types in two of three experiments, but displayed a preferential and significant (p = 0.020) trend towards neutrophil chemokinesis in experiment **5.2.10** (Table 5.1). For *O. ochengi*, although the neutrophil chemotactic index remained relatively constant over the three experiments for male worms [**5.2.9**, **5.2.10**, **5.2.11** *(a)*] at between 2 and 4, the overall neutrophil migration score for *Wolbachia*-positive extracts was only significantly higher than that for eosinophils in experiment **5.2.11** (p = 0.001); while eosinophil chemotaxis was significantly greater than that for neutrophils in experiment **5.2.10**, irrespective of *Wolbachia* status (Table 5.1). For female worms, one experiment showed higher levels of overall eosinophil migration compared with neutrophils, irrespective of *Wolbachia* content (p = 0.001); whereas chemotaxis of eosinophils was significantly greater than that for neutrophils towards *Wolbachia*-depleted extracts in two experiments (Table 5.1).

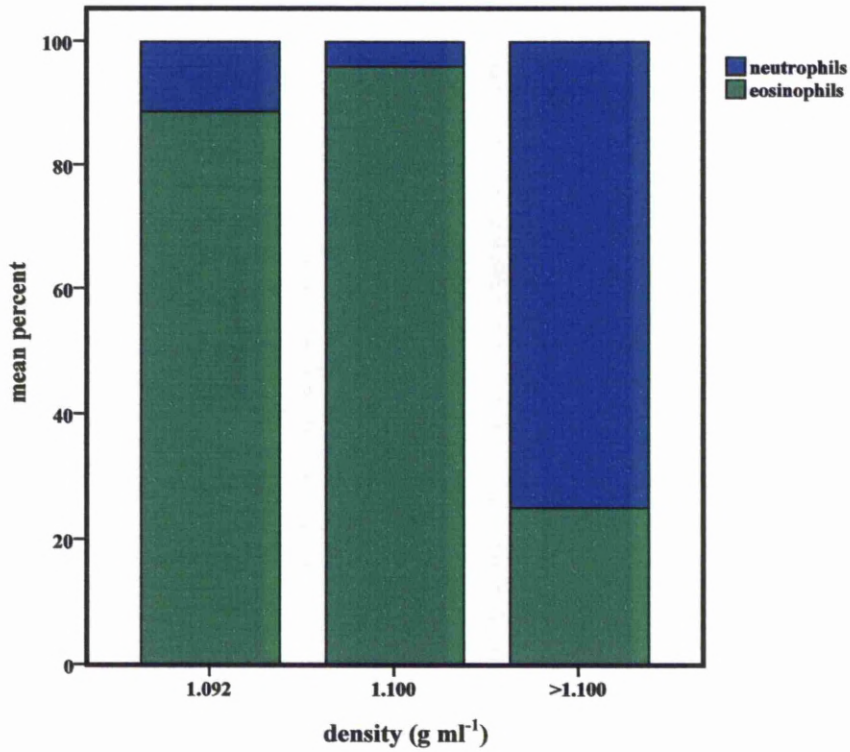
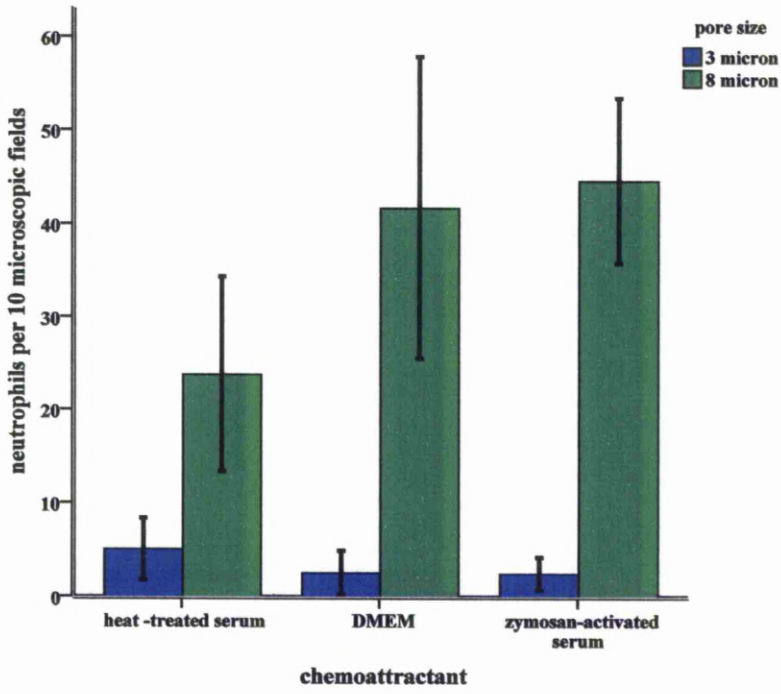


Figure 5.2.1. Separation of bovine granulocytes using a Percoll® discontinuous density gradient. Data represent mean percent granulocyte composition ($n = 6$)

.

(a)



(b)

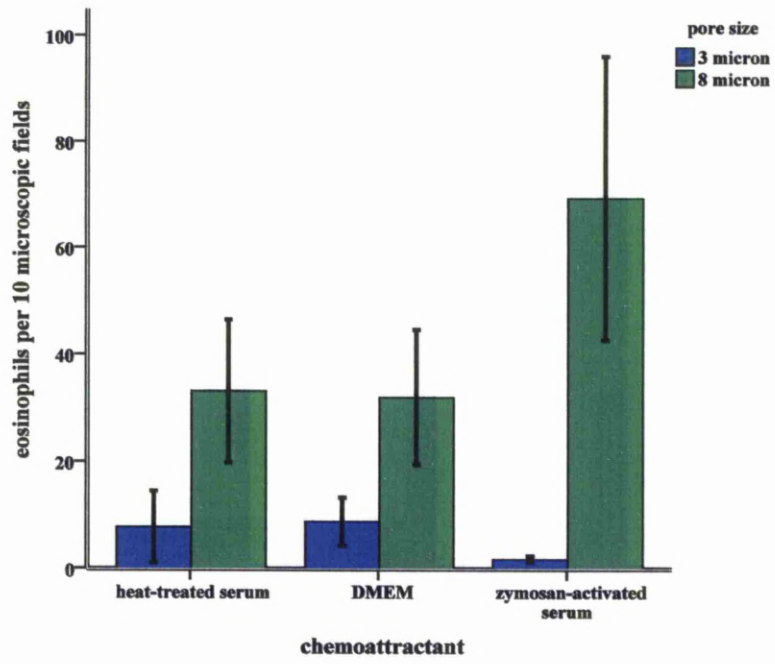


Figure 5.2.2. Neutrophil *(a)* and eosinophil *(b)* migration through polycarbonate membranes containing either 3 micron or 8 micron pores (Nunc cell culture inserts, ThermoFisher Scientific), towards DMEM, DMEM containing 1% heat-treated serum or DMEM containing 10% zymosan-activated serum. Data represents mean ± 1 SE ($n = 6$).

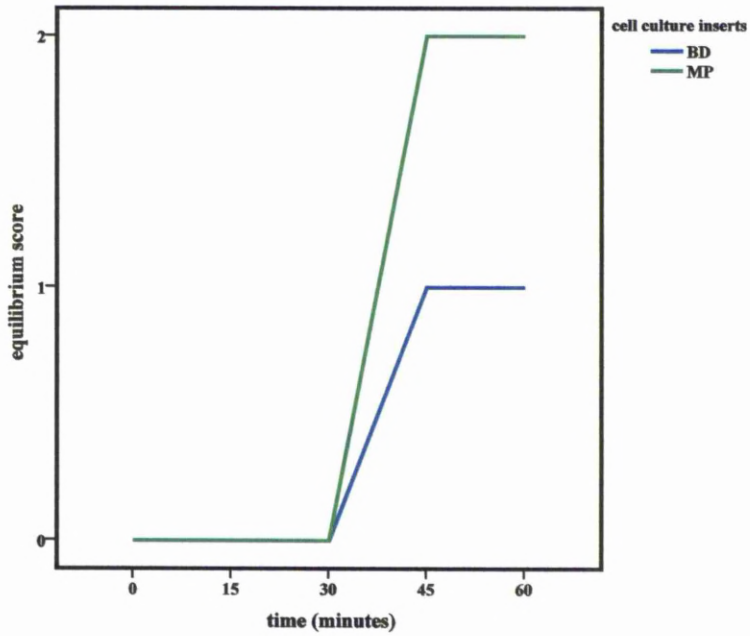


Figure 5.2.3. Time of diffusion of trypan blue in DMEM culture medium, through 8 micron cell culture inserts from Becton Dickinson (BD) or Millipore (MP). Equilibrium was scored by visual comparison. Range: 0 = no visible diffusion; 1 = partial diffusion; 2 = maximum diffusion (but not complete equilibrium) into upper chamber.

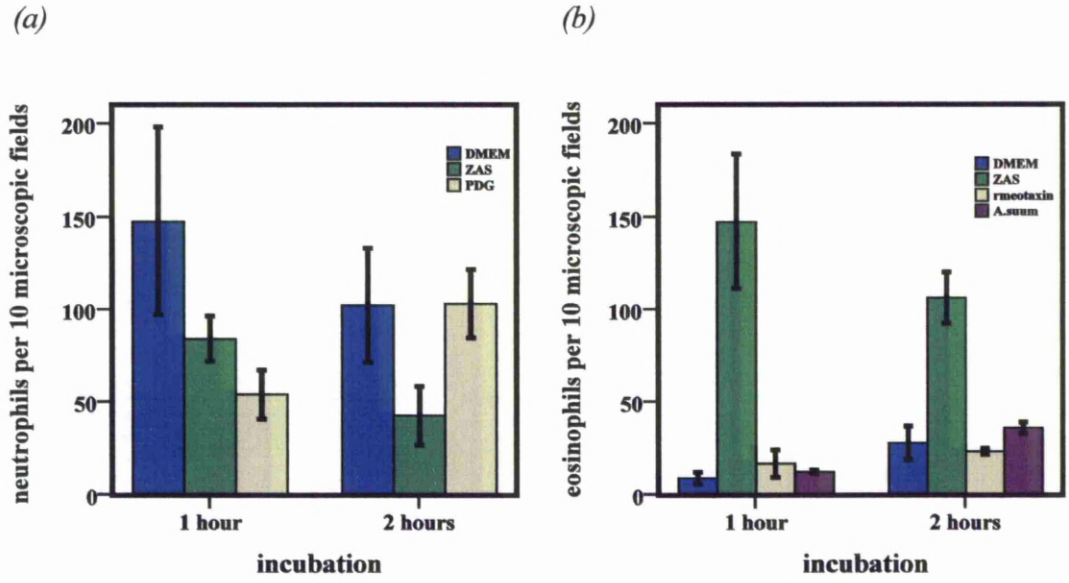


Figure 5.2.4. Bovine neutrophil *(a)* and eosinophil *(b)* migration towards chemoattractants after 1 or 2 hours. Granulocyte ratios of 99% neutrophils:1% eosinophils *(a)*, or 16% eosinophils:84% neutrophils *(b)*, were used at a concentration of 1 $\times 10^6$ cells per well. All chemoattractants were diluted in DMEM cell culture medium containing 1% heat-treated serum. Chemoattractentrs were: DMEM: Dulbecco's Modified Eagle Medium; ZAS: 10% zymosan-activated serum; PDG: 3 μg ml^{-1} peptidoglycan; rmeotaxin: 10 ng ml^{-1} recombinant murine eotaxin; 0.1 μg ml^{-1} *A.suum*. Bars show mean cell migration ± 1 SE (n = 4).

Figure 5.2.5, Figure 5.2.6 and Figure 5.2.7 are positive control data from three individual experiments.

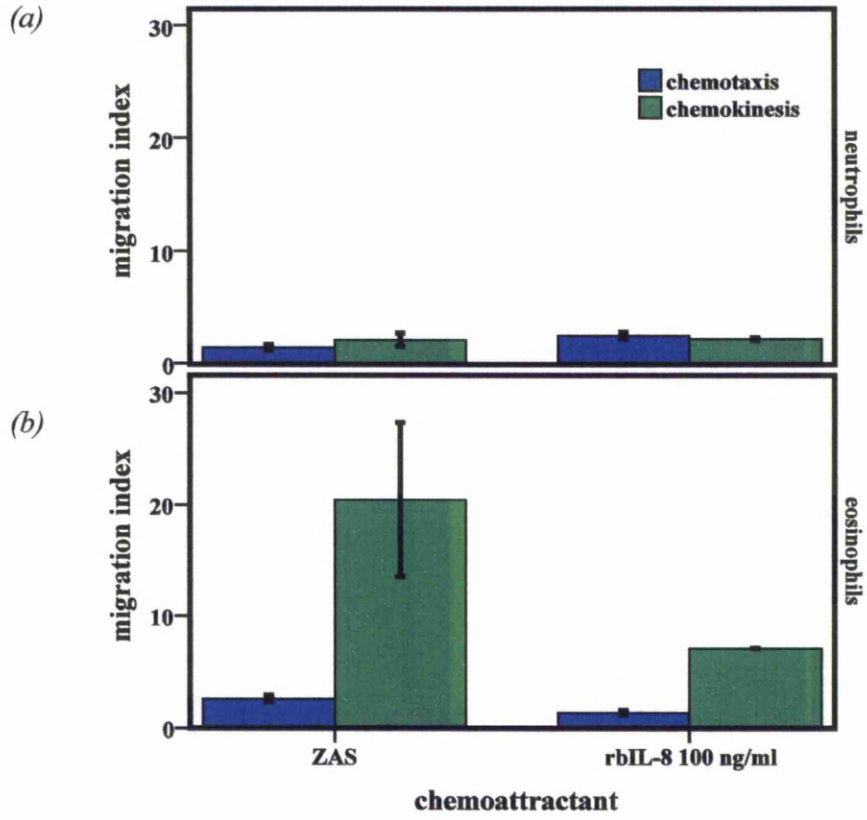


Figure 5.2.5. Chemotactic (directional movement) and chemokinetic (random movement) migration indices for bovine neutrophils *(a)* and eosinophils *(b)* towards 10% zymosan-activated serum (ZAS), or 100 ng ml $^{-1}$ recombinant bovine IL-8 (rbIL-8) in DMEM cell culture medium supplemented with 1% heat-treated serum. Migration indices were calculated by dividing the number of cells migrated in the presence of the chemoattractant by the number of cells migrated in DMEM alone. The granulocyte ratio was 40% neutrophils:60% eosinophils at a concentration of 5 $\times 10^6$ cells ml^{-1}. Bars show mean migration index ± 1 SE for chemotaxis (n = 6) and chemokinesis (n = 4).

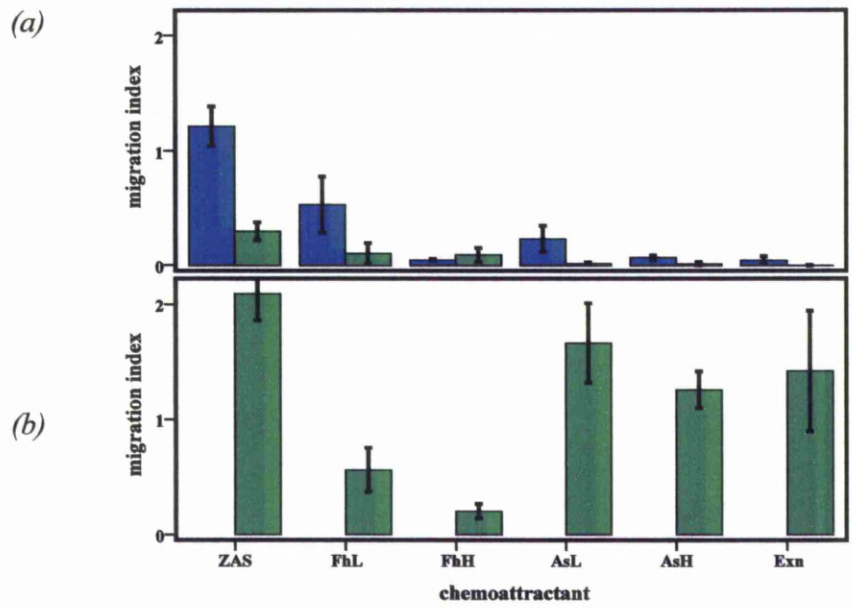


Figure 5.2.6. Chemotactic (directional movement) migration indices for bovine neutrophils *(a)* and eosinophils *(b)* towards various extracts. Migration indices were calculated by dividing the number of cells migrated in the presence of the chemoattractant, by the number of cells migrated in DMEM alone. Cell populations (5×10^6 cells ml^{-1}) were 100% neutrophils (blue bars) or eosinophil enriched [(59%), green bars]. Extract key: FhL, *F. hepatica* 1 µg ml^{-1}; FhH, *F. hepatica* 10 µg ml^{-1}; AsL, *A. suum* 1 µg ml^{-1}; AsH, *A. suum* 10 µg ml^{-1}; Exn, recombinant murine eotaxin 100 ng ml^{-1}. All chemoattractants were diluted in DMEM cell culture medium supplemented with 1% heat-treated serum. Error bars show mean migration index ± 1 SE ($n = 4$).

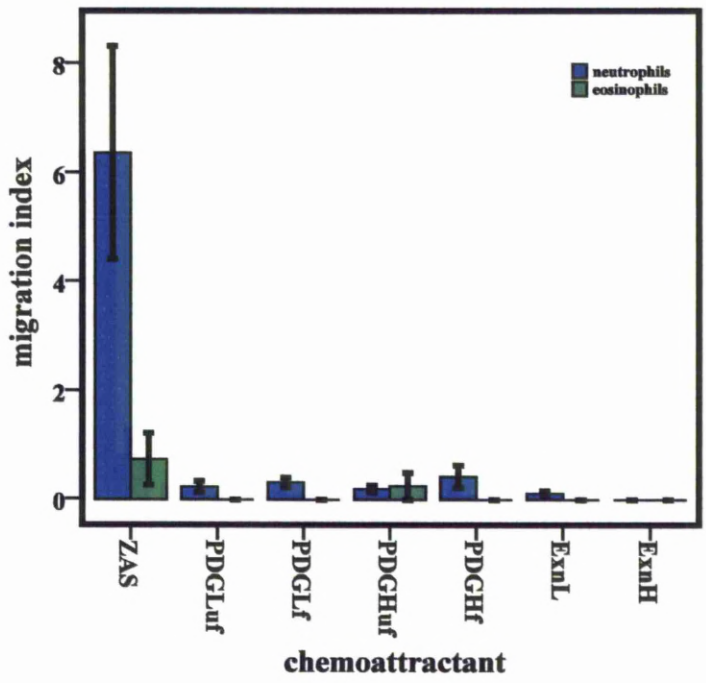


Figure 5.2.7. Chemotactic (directional movement) migration indices for bovine neutrophils and eosinophils towards zymosan-activated serum (ZAS), peptidoglycan (PDG), and *rm*eotaxin. Migration indices were calculated by dividing the number of cells migrated in the presence of the chemoattractant, by the number of cells migrated in DMEM alone. The granulocyte ratio was 97% neutrophils:3% eosinophils at a concentration of 5×10^6 cells ml^{-1}. All chemoattractants were diluted in DMEM cell culture medium supplemented with 1% heat-treated serum. Chemoattractant key: PDGLuf, unfiltered PDG 0.3 μ ml^{-1}; PDGLf, filtered PDG 0.3 μg ml^{-1}; PDGHuf, unfiltered PDG 3 μg ml^{-1}; PDGHf, filtered PDG 3 μg ml^{-1}; ExnL, *rm*eotaxin 25 ng ml^{-1}; ExnH, *rm*eotaxin 100 ng ml^{-1}. Bars show mean migration index ± 1 SE ($n = 4$).

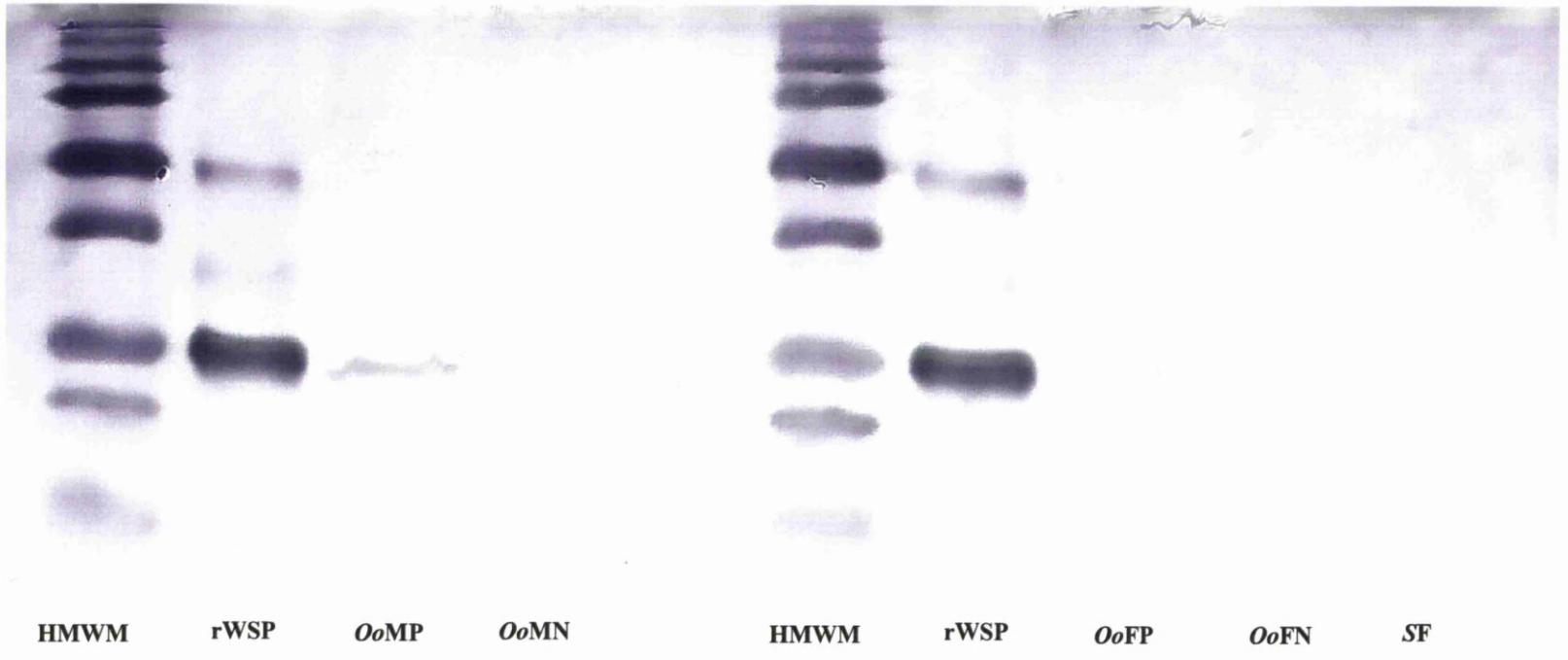
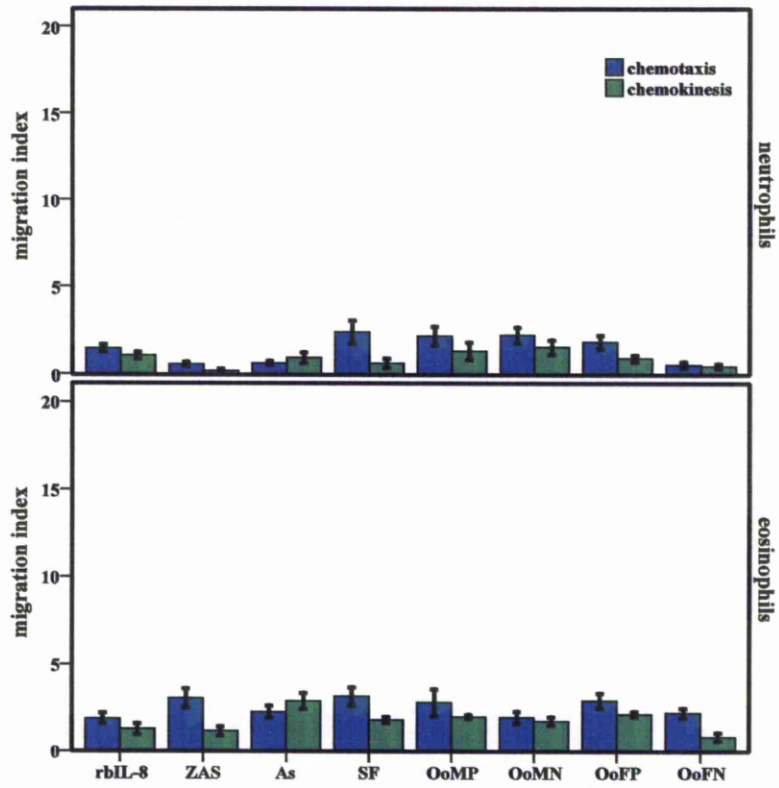


Figure 5.2.8. Identification of *Wolbachia* surface protein (WSP) by Western blot at the expected molecular weight of 24 kDa. Rabbit Anti-WSP antibody was used at 1 in 400 dilution, followed by goat anti-rabbit IgG-alkaline phosphatase secondary antibody at 1 in 30,000 dilution, before development in 1-Step™ nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3`-indolyphosphate p-toluidine salt solution (Thermo Scientific), diluted 1:1 in water. The 25 kDa band of the high molecular weight markers [HMWM (range 10 – 250 kDa)] is indicated. Key: HMWM, high molecular weight marker; rWSP, recombinant *Wolbachia* surface protein; *Oo*MP, *Wolbachia*-positive *O. ochengi* male; *Oo*MN, *Wolbachia*-negative *O. ochengi* male; *Oo*FP, *Wolbachia*-positive *O. ochengi* female; *Oo*FN, *Wolbachia*-negative *O. ochengi* female; SF, *S. labiatopapillosa* female.

5.2.9

(a)

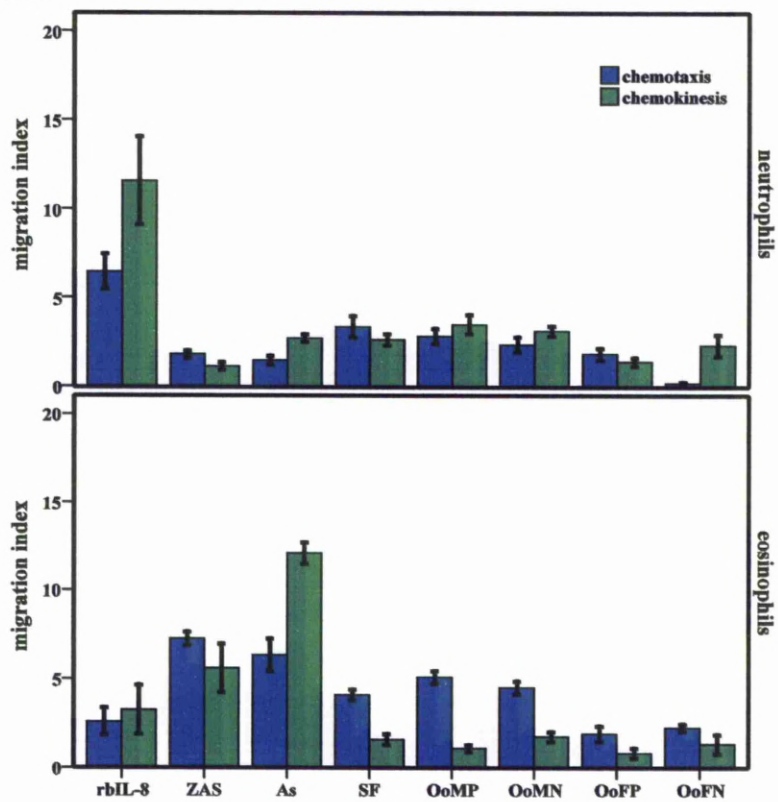
(b)



5.2.10

(a)

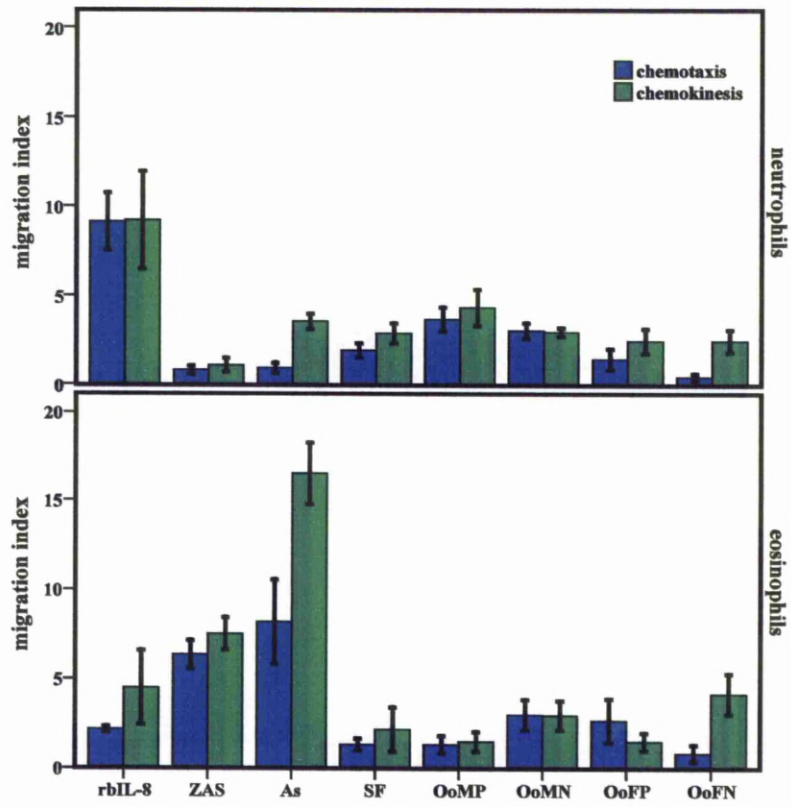
(b)



5.2.11

(a)

(b)



Figures 5.2.9; 5.2.10; 5.2.11. Bovine neutrophil *(a)* and eosinophil *(b)* responses to the neutrophilic positive control, recombinant bovine IL-8 [(rbIL-8), 100 ng ml^{-1}]; the eosinophilic positive controls zymosan-activated serum [(ZAS), 10%] and *Ascaris suum* extract [As (10 μg ml^{-1})]; and filarial extracts from female *Setaria labiatopapillosa* (SF), *Wolbachia*-depleted male (OoMN) and female (OoFN) *Onchocerca ochengi*, and *Wolbachia*-replete male (OoMP) and female (OoFP) *O. ochengi*. All filarial extracts were used at a concentration of 10 μg ml^{-1}. All chemoattractants were diluted in DMEM cell culture medium supplemented with 1% heat-treated serum. Granulocyte ratios were 50% neutrophils:50% eosinophils in **5.2.9** and 57% neutrophils:43% eosinophils in **5.2.10** and **5.2.11** (total cell concentration, 5×10^6 ml^{-1}). Bar shows mean migration index ± 1 SE (n = 6) for chemotaxis or chemokinesis (except in **5.2.9**, where n = 4 for chemokinesis data only).

Table 5.1. Trends and interactions of granulocyte migration for definitive experiment set **5.2.9**; **5.2.10**; and **5.2.11**.

	Cell type, main effect (trend and *p* value)			**Migration type, main effect difference (trend and *p* value)**			**Interaction between cell type and migration (trend and *p* value)**		
	Experiment								
Chemoattractant	**5.2.9**	**5.2.10**	**5.2.11**	**5.2.9**	**5.2.10**	**5.2.11**	**5.2.9**	**5.2.10**	**5.2.11**
ZAS	**E>N <0.001**	**E>N <0.001**	**E>N <0.001**	**CT>CK 0.008**	**CT>CK 0.034**	0.363	0.061	0.785	0.889
***rb*IL-8**	0.359	**N>E 0.001**	**N>E 0.002**	0.094	0.248	0.901	0.792	0.541	0.469
A. suum	**E>N <0.001**	**E>N <0.001**	**E>N <0.001**	0.158	**CK>CT 0.001**	**CK>CT <0.001**	0.623	0.310	0.764
S. labiatopapillosa	0.108	0.421	0.120	**CT>CK 0.012**	**CT>CK 0.001**	0.590	0.695	**CK N>E 0.020**	0.537
***O. ochengi* male, *Wolbachia*-positive**	0.343	0.142	**N>E 0.001**	0.202	**CT>CK <0.001**	0.725	0.982	**CT E>N CK N>E <0 001**	0.895
***O. ochengi* male, *Wolbachia*-negative**	0.845	0.656	0.512	0.278	**CT>CK 0.013**	0.924	0.556	**CT E>N CK N>E <0.001**	0.904
***O. ochengi* female, *Wolbachia*-positive**	**E>N 0.008**	0.259	0.853	**CT>CK 0.035**	**CT>CK 0.021**	0.712	0.851	0.234	0.240
***O. ochengi* female, *Wolbachia*-negative**	**E>N 0.001**	0.079	0.255	**CT>CK 0.006**	0.171	**CK>CT <0.001**	**CT E>N 0.013**	**CT E>N 0.001**	0.578

Bold type indicates statistical significance at $p < 0.05$.
Key: E, eosinophils; N, neutrophils; CK, chemokinesis; CT, chemotaxis.
The centre panel is shaded to aid viewing.

5.3. Discussion

5.3.1. Determination of assay parameters

In recent publications, the separation of bovine neutrophils from peripheral blood has been generally well described, but in many of these reports, the presence of eosinophils was regarded as a necessary contamination of otherwise pure populations of neutrophils. For example, eosinophils were seen in small numbers after the isolation of bovine neutrophils using biomagnetic beads (Soltys et al., 1999), but no further attempts were made to isolate eosinophils in the cell mixture. For our experiment, a 1:1 neutrophil:eosinophil ratio was sought to ensure a consistent cellular composition, where the effect of chemoattractants on eosinophils would be apparent, since the natural granulocyte ratio in peripheral blood (*i.e.*, a very small proportion of eosinophils) may have masked any subtle effects. Although the successful and aseptic separation of bovine eosinophils has been described in older publications (Chambers et al., 1985; Riding and Willadsen, 1981), the method of collection and the reagents used throughout these procedures prevented scale-up within our laboratory.

Asepsis was also an important consideration for the experiments to avoid the aberrant migration of neutrophils towards contaminating bacterial products. Freiburghaus and Jorg (1990) reported the successful purification of bovine eosinophils using 6 litres of fresh blood. Although these workers did not perform their protocol under aseptic conditions and we lacked the equipment to process this quantity of blood, we were able to withdraw 1 litre of blood aseptically using an adapted procedure used for blood transfusion purposes (Soldan, 1999). The initial centrifugation procedures were then scaled-down to obtain a large pellet of bovine

granulocytes, from which eosinophils and neutrophils were successfully separated using a Percoll® discontinuous density gradient (Chambers et al., 1983). Using this hybrid method, we were consistently able to produce granulocyte populations containing sufficiently high numbers of eosinophils to seed >100 cell culture wells during the definitive experiments.

Bovine neutrophils were found to migrate through 5 μm pores after 30 minutes of incubation with bovine IL-8-containing bronchio-alveolar lavage fluid from *Mannheimia haemolytica*-infected calves (Caswell et al., 2001). Hallen-Sandgren and Bjork (1988) used 3 μm Millipore™ filters to quantify chemotaxis of bovine neutrophils towards complement-activated serum, and Chambers (1985) used 5 μm Millipore™ filters within a Boyden chamber for assays of bovine eosinophil chemotaxis. However, in a more recent study using ovine eosinophils, 8 μm pores were used in modified Boyden chambers to assess chemotaxis towards *T. circumcincta* and *H. contortus* (Wildblood et al., 2005). In our initial experiments, 3 μm pores were found to be too small for quantifiable chemotaxis (Figure 5.2.2), and thus inserts containing 8 μm pores were selected. The pore size data were collected from an assay using Nunc™ cell culture inserts with polycarbonate membranes; however, subsequent evaluation of polyethylene terephthalate membranes determined that they had superior mechanical properties to polycarbonate, and this led to the decision to use Becton Dickinson Falcon™ cell culture inserts and plates. These were then superseded by Millipore™ Millicell cell culture plates to enable numerous assays to be conducted simultaneously, as this system required smaller working volumes.

Cell migration assay times vary considerably between published protocols. Assays using human neutrophils and IL-8 performed over 3 hours reported that cell migration increased over 30 minutes but plateaued by 60 minutes (Erger and Casale, 1995), and canine neutrophils migrated towards WSP within 1 hour of incubation (Bazzocchi et al., 2003). Bovine neutrophils became activated by ZAS (via shape change) within 5 - 10 minutes (Forsell et al., 1985) and by IL-8 within seconds or minutes, followed by migration within 30 minutes (J. Caswell (1999); and personal communication). In contrast, bovine eosinophils migrated towards ZAS over 1.5 hours (Chambers et al., 1985). Additional consideration was given to the angle of the slope of the chemotactic gradient, which is reported to influence the orientation of neutrophils (Zigmond, 1977), and the possibility of significant cell death, which may occur when incubation times of over 60 minutes are used in neutrophil assays (Zigmond, 1977).

Our data showed that with the Millipore and BD systems, there was no visible diffusion of trypan blue into the upper wells, indicating that a steep chemotactic gradient was present for the first 30 minutes (Figure 5.2.3). At 45 minutes the trypan blue had diffused further into the upper well of the Millipore system, hence the angle of the chemoattractant gradient was lower, although complete equilibrium had not been reached. In the cell migration assay, the number of cells that had migrated after one hour of incubation was observed to decrease by two hours, which suggests that cell migration had peaked and cell death was beginning to have an impact by this time (Figure 5.2.4). This is consistent with a possible loss of cell adhesiveness, causing cells to fall into the lower chamber. Thus, the 1 hour incubation time was chosen as the optimum period to ensure a steep chemoattractant gradient while also limiting the possibility of cell loss.

5.3.2. Determination of positive controls

Recombinant bovine IL-8 (*rb*IL-8) was selected for neutrophils, and ZAS and *A. suum* extracts were selected for eosinophil positive controls, respectively. Caswell *et al.* (1999) characterised *rb*IL-8 as a neutrophil-specific chemokine via *in-vivo* and *in-vitro* assays. In their *in-vitro* studies, *rb*IL-8 induced dose-dependent migration and activation of neutrophils, evidenced by shape change and development of pseudopodia. In contrast, eosinophil migration was rare, even when an increased eosinophil ratio was used (Caswell et al., 1999). These eosinophil data apparently contradict another report that showed eosinophil migration through synthetic 3 μm pores and through endothelium and epithelium *in-vitro*, occurring by 60 minutes after exposure to IL-8 (Erger and Casale, 1995). However, this response may only occur with eosinophils previously primed with IL-5 (Schweizer et al., 1994). Low numbers of eosinophils were also found to migrate into the ovine dermis following instillation of recombinant ovine IL-8 (Seow et al., 1994). In our study, positive control experiments showed that *rb*IL-8 elicited a similar chemotactic and chemokinetic effect to ZAS on neutrophils (migration index, ~2). In addition, two out of three definitive experiments indicated a significantly greater response to this chemokine in neutrophils relative to eosinophils (Table 5.1).

Peptidoglycan was selected as another potential neutrophil positive control for neutrophil migration because *Wolbachia* is known to synthesis peptidoglycan precursors (Henrichfreise et al., 2009). Peptidoglycan was demonstrated to specifically activate human neutrophils in a dose-dependent manner *in-vitro* via the TLR-2 pathway (Mattsson et al 2003); however, a very recent publication has provided evidence to the contrary (Iyer and Coggeshall, 2011). Two concentrations of PDG; (both 0.2 μm-filtered or unfiltered preparations were assessed, as polymeric

insoluble particles may be more stimulatory (Barotciorbaru et al., 1985) were also evaluated as a positive control candidate for neutrophils, but did not cause as great an effect as *rb*IL-8. Although bovine neutrophils store and secrete a PDG recognition protein that has been demonstrated to mediate the lysis of heat-killed bacteria (Tydell et al., 2006), the chemoattractive properties of PDG have not been elucidated in this species.

Zymosan-activated serum has been shown to induce both bovine neutrophil (Caswell et al., 1999) and eosinophil (Chambers et al., 1985) migration *in-vitro*. Our positive control data showed that ZAS had a greater effect on eosinophils than neutrophils in two out of three preliminary experiments (Figure 5.2.5; Figure 5.2.6), and in all of the definitive experiments, this difference was significant (Table 5.1).

Ascaris suum was shown to attract both eosinophils and neutrophils in to the bovine udder within 6 hours of instillation (Grewal and Babiuk, 1979), but the presence of neutrophils was thought to have been due to endotoxin present within the *A. suum* extract used. Although we did not test for endotoxin in any of our reagents, we found that *A. suum* was a consistent and potent positive control for eosinophils and had relatively little effect on neutrophils (Figure 5.2.6; **5.2.9**; **5.2.10**; **5.2.11**; Table 5.1).

Recombinant murine eotaxin was used as a positive control for the *in-vitro* chemotaxis of ovine eosinophils by Wildblood et al. (2005), inducing a ~1 log increase in eosinophil migration over the negative control when used at 100 ng ml^{-1}. Our positive control data demonstrated a similar response of bovine eosinophils towards *rm*eotaxin, *A. suum* and ZAS in one experiment [Figure 5.2.6 *(b)*], but not in a subsequent experiment, unlike ZAS that exhibited more consistent activity (Figure 5.2.7).

5.3.3. Granulocyte chemotaxis and chemokinesis in the presence of filarial extracts

Early chemotaxis studies determined that natural *O. volvulus* extracts were chemotactic (and to a lesser extent, chemokinetic) for human neutrophils, and that the effect was dose-dependent (Rubio de Kromer et al., 1998). Fractionation of the extract revealed two components that displayed chemotactic activity (with a threshold index >2), one with a high (>200 kDa) molecular mass and the other representing a very small protein or mixture (<12 kDa). Subsequently, *O. volvulus* extracts containing *Wolbachia* induced neutrophil chemotaxis *in-vitro*, in contrast to *Wolbachia*-depleted worm extracts; this was also dose dependent, and peaked at a parasite protein concentration of 100 μg ml^{-1} (Brattig et al., 2001). The results from our study indicate that normal adult *O. ochengi* extracts are also chemotactic and chemokinetic for bovine neutrophils, to a similar degree as shown in the report by Rubio de Kromer [median chemotactic index ~2 in response to 10 μg ml^{-1} extract (1998)]. However, *Wolbachia*-depleted worm extracts [confirmed via Western blot, Figure 5.2.8)] did not display consistently different properties with regard to the chemotaxis of neutrophils or eosinophils (Figures 5.2.9; 5.2.10; 5.2.11; and Table 5.1). Nonetheless, the definitive experiment data did show some commonalities between the responses to filarial extracts (Table 5.1). There were trends towards greater overall chemotaxis than chemokinesis (*i.e.*, across both cell types) for *S. labiatopapillosa* and *Wolbachia*-positive female *O. ochengi*. For *Wolbachia*-negative female *O. ochengi*, eosinophils tended to exhibit higher chemotactic indices than did neutrophils. A Zigmond-Hirsch checkerboard analysis (Zigmond and Hirsch, 1973) may have further elucidated the dynamics between chemotaxis and chemokinesis in response to the different chemoattractants.

The expected result of a switch from neutrophilic to eosinophilic migration in the *Wolbachia* depleted assays (*i.e.*, mirroring our *in vivo* data, chapter 6) was not evident. The reasons for the inconsistency in these results as well as the apparent contradiction with the study by Brattig et al (2001) could be due to a variety of factors. To decrease biological variation between assays, a single donor animal was used and blood was collected at the same time of day and via a consistent protocol. The selected donor animal was male to avoid granulocyte changes associated with oestrus. However, despite procedures to limit natural variation, blood parameters do change over time and this may be due to environmental factors (e.g. parasitic load, subclinical infections) or physiological fluctuations. *In-vivo* granulocyte priming from external factors could cause activation of neutrophils or eosinophils during the process of cell separation; however, this was not observed during viability tests, and an increase in the migration index in response to positive controls compared to negative controls suggests that the granulocytes had not lost an ability to respond further *in vitro*. In our experiments a high starting concentration of eosinophils was used, compared to the physiological concentration present *in vivo*. This was to ensure that in laboratory conditions, any effect on eosinophil chemotaxis would be apparent in the short incubation times, and to reduce the risk of "crowding-out" by neutrophils. However, because of the unknown effects that an unnaturally high concentration of one cell type may have on other cells within the system (from physical or cell signalling influences), this should be recognised as a necessary compromise within the *in-vitro* experiment.

There are also important differences between our study and that conducted by Brattig (2001). In Brattig's study, filariae were depleted of *Wolbachia* by a 6-week doxycycline treatment, and onchocercomata were removed 4 and 9 months after the

end of treatment. This is in contrast to a 2-week, short intensive oxytetracycline regimen administered to *O. ochengi*-infected cattle in our study, followed by the removal of onchocercomata at 10 - 12 weeks post-treatment for our migration assays. The two regimens both produce *Wolbachia*-depleted filariae; however, the residual level of bacteria and the composition of the worms' tissues are likely to vary due to the differing lengths of treatment and the interval before nodulectomy. We specifically chose the 2-week regimen because although the worms become heavily depleted of *Wolbachia*, they can be unequivocally defined as fully viable, since Gilbert et al. (2005) demonstrated that *Wolbachia* recrudesce at 24 weeks following this treatment and the worms retain normal motility and metabolism. In addition, Brattig only used female *O. volvulus* for chemotaxis experiments (Brattig et al., 2001), which are likely to retain a considerable residue of host material on their surface, and this could play a significant role in granulocyte modulation. Another key difference between our experiments and those of Brattig (2001) was that we only used non-gravid female worm preparations for both the *Wolbachia*-positive and –depleted extracts, rather than worms of uncharacterised reproductive status. We sought to control for gravidity to avoid two potentially confounding effects on our data. Firstly, since eosinophils have been found in association with microfilariae in *O. volvulus* onchocercomata (Wildenburg et al., 1996), the inclusion of intrauterine microfilarial extracts in our assays could have skewed the results. Secondly, tetracycline antibiotics lead to reduced levels of intrauterine microfilariae in adult females (Bandi et al., 1999; Hoerauf et al., 2000a; Hoerauf et al., 2000b; Langworthy et al., 2000), and thus effects attributed to *Wolbachia* depletion could actually result from reduced levels of embryonic stages or their degeneration.

The concentration of chemoattractants can also influence the migration index of cells, as a low concentration can have little effect, whereas too high a concentration can cause cell stasis (Heit et al., 2002). The WSP from *D. immitis Wolbachia* stimulated significant neutrophil chemokinesis, which reached a peak at a concentration of 25 ng ml^{-1} (Bazzocchi et al., 2003). However, the use of crude parasite extracts seems to require a much higher concentration in order to elicit granulocyte migration. In a study using extracts of *Strongyloides stercoralis*, murine eosinophil migration was not different from the control wells until a concentration of 1 mg/ml extract was used (Stein et al., 2009). For *O. volvulus* extracts, significant differences were reported between *Wolbachia*-replete and depleted worms at concentrations of 100 μg ml^{-1} and 150 μg ml^{-1}, while a migration index difference of ~1 was still evident at a concentration of 20 μg ml^{-1} (Brattig et al., 2001). A neutrophil migratory index range between 1.3 and 3.8 was reported in response to *Wolbachia*-replete *O. volvulus* for concentrations between 5×10^{-4} and 500 μg ml^{-1} (Rubio de Kromer et al., 1998) . In our preliminary experiments, we found that the neutrophil migration index towards *O. ochengi* extracts were between 1 and 1.5 for concentrations of 10, 50 and 100 μg ml^{-1} (data not shown).

5.3.4. Considerations for further investigation

Although the quantification of migrated cells adherent to the underside of the cell culture insert membrane is an accepted method for measuring chemotaxis and chemokinesis, Zigmond and Hirsch (1973) proposed that measuring chemotaxis via the leading edge method was more reliable, as this prevented cell detachment from the underneath of the membrane. Although we were satisfied that cell migration indices were not affected by cell loss, future work could compare these quantification methods to identify possible discrepancies.

To further reproduce the *in-vivo* environment, migration assays using granulocytes and serum from *O. ochengi*- infected animals could be performed. Cells and immune serum from infected animals are likely to possess immunological factors that would influence cell migration and more closely reproduce *in-vivo* conditions. However, King et al., (1983) reported that there was no difference in the chemotaxis of neutrophils from *O. volvulus* infected patients compared with uninfected individuals, and Rubio de Kromer (1998) reported significant chemotactic indices when using neutrophils from uninfected patients. Furthermore, the limited availability of laboratory equipment at the Ngaoundéré field site in Cameroon severely limits the application of the cell separation protocol for migration assays using blood from *O. ochengi*-infected cattle. An alternative would be to collect immune serum and cells from UK cattle infected with *O. lienalis* or *O. gutturosa*; however, the fact that these filariae are not nodule-forming and thus not directly analogous to *O. ochengi* and *O. volvulus* could limit the relevance of this approach.

Male *O. ochengi* worms were included in our assays to exclude the potential effects of residual host material that adheres tightly to the cuticle of female worms. To further limit the possibility of mammalian contamination, and to specifically test granulocyte migration towards *Wolbachia* and their products, bacterial cells could be purified from insect cell-lines or filarial worms, as has been demonstrated in other studies (McGarry et al., 2004).

6. The effects of treatment with oxytetracycline of cattle on the cellular composition of *Onchocerca ochengi* nodules.

6.1. Introduction

Ex-vivo nodule cell population dynamics were studied within nodules removed during the longitudinal experiment, using cattle groups treated with macrofilaricidal regimens of either oxytetracycline or melarsomine (Cymelarsan®), and compared with untreated controls.

Previous work has elucidated that in the untreated individual, vast accumulations of neutrophils surround adult filariae within *O. volvulus* (Brattig et al., 2001) and *O. ochengi* (Wildenburg et al., 1997) nodules. *In-vitro* chemotaxis assays facilitated the demonstration of neutrophilic chemotaxis towards female *O. volvulus* extracts, which as they were untreated, would have contained *Wolbachia* (Rubio de Kromer et al., 1998). Neutrophils were then demonstrated to be specifically attracted to *Wolbachia*, as evidenced by the lack of neutrophilic accumulation in the vicinity of worms removed from patients who had previously received a 6-week chemotherapeutic regimen of doxycycline (100 mg/day), thus resulting in the elimination of viable *Wolbachia* (Brattig et al., 2001).

The first quantitative analysis of cell populations within *O. ochengi* nodules removed from cattle receiving a non-macrofilaricidal SIR, or macrofilaricidal COM regimen of oxytetracycline revealed a stark shift in cell populations over time (Nfon et al., 2006), which were temporally correlated with the depletion and repopulation of *Wolbachia* within worms observed in an earlier study (Gilbert et al., 2005). Taken together, these experiments suggest that the replacement of neutrophils with an

influx of eosinophils is due to a decline in *Wolbachia* rather than as a consequence of filarial damage. However, as only antibiotics were used in these experiments, the dynamics of cell populations during macrofilaricidal activity induced by a non-antibiotic drug remained uncharacterised. Furthermore, because few time points were analysed, the study of Nfon et al. (2006) did not provide a comprehensive timeline of cell dynamics.

The current experiment sought to give further insight into the cell population shifts in nodules from animals that received a macrofilaricidal regimen of oxytetracycline, in comparison to nodules from animals receiving melarsomine (see Section 3.2); a drug which was demonstrated to have no specific effect on *Wolbachia* (see Section 3.1). This was achieved by using larger groups of animals (n = 6) in comparison to previous studies (n = 3 - 6), and more frequent nodulectomies [9 time-points, compared to Nfon et al. (2006) with 4 and Gilbert et al. (2005) with 6]. Whole nodule transects (see Section 3.2.4) were studied microscopically and granulocytes were identified and digitally labelled to ensure accurate quantification.

6.2. Results

6.2.1. Effect of treatment on nodule granulocyte populations

Digital stitching of nodule sections facilitated evaluation of the nodule cross-section as a whole (Figure 6.2.1), prior to individual digital tagging of neutrophils and eosinophils (illustrated in Figure 6.2.2). Nodules from the control group consistently contained many sections of viable filariae (Figure 6.2.1) surrounded by a cellular infiltrate typical of *O. ochengi* [Figure 6.2.3 *(a)*], which were centrally dominated by neutrophils. Many of these cells were seen in close proximity or attached to worm

cuticles, or intermingled with intensely eosinophilic material containing antibody-antigen complexes and tissue debris, known as Splendore-Hoeppli deposits (Hussein, 2008) [Figure 6.2.3*(b)*]. Absolute granulocyte quantification revealed that neutrophils consistently outnumbered eosinophils in this group (overall median ratio = 0.004) which did not change significantly throughout the experiment (Friedman test: $\chi^2 = 5.6$, $p = 0.507$). Eosinophils were typically scarce, if present at all, and were most commonly located around the periphery of the neutrophilic infiltrate [Figure 6.2.3 *(a)*].

A similar scenario was observed in oxytetracycline-treated nodules removed two weeks post-treatment (wpt), where healthy female worms containing microfilariae were surrounded by a rich neutrophilic infiltrate [Figure 6.2.4 *(a)*]; and also at 4 wpt, where neutrophils far outnumbered any other cells, including macrophages (a qualitative observation, as digital quantification of macrophages was outside the scope of this study) [Figure 6.2.4 *(b)*]. However by 8 wpt, absolute quantification revealed a decreasing trend in the neutrophil density [Figure 6.2.6 *a* (iii)], and eosinophilic infiltrates were observed in the vicinity of worm cuticles [Figure 6.2.4 *(c)*]. At 12 wpt, digital quantification within transects revealed an increase in eosinophils [Figure 6.2.6 *b* (iii)], which were found forming layers adjacent to worms [Figure 6.2.3 *(c)* and *(d)*] and furthermore, were sometimes observed attached to the worm cuticle [Figure 6.2.4 *(d-f)*]. By 36 wpt, relative eosinophil numbers peaked [Figure 6.2.6 *b* (iii)], evidenced by a highly significant 275-fold increase of the eosinophil:neutrophil ratio [Figure 6.2.6 *c* (iii); (Friedman test: $\chi^2 = 19.3$, $p < 0.001$)]. Macrophages were also present during this time [Figure 6.2.4 *(d)* and *(e)*], and from 48 wpt, the nodule cytology assumed the appearance of a wound-healing response, characterised by the infiltration of fibroblasts, macrophages, lymphocytes,

and the retention of moderate numbers of eosinophils. This was accompanied by worm disintegration and nodule resolution [Figure 6.2.4 *(g)* and *(h)*].

In the melarsomine group, eosinophils were observed in nodule transects more frequently than in the control group throughout the time course (Figure 6.2.5), and a modest increase was observed especially in nodules that were resolving [Figure 6.2.5 *(g)*], resulting in a slight upward trend in the eosinophil: neutrophil ratio [Figure 6.2.6 *c* (ii)]. However, this was not statistically significant (Friedman test: $\chi^2 = 6.0$, p = 0.473), and eosinophils were less commonly observed in layer formations or adherent to the worm cuticle relative to the oxytetracycline group. The cellular composition was mixed [Figure 6.2.5 *(a)*], and the heavy neutrophilic infiltrate [Figure 6.2.5 *(b)*] decreased overall from 4 wpt to reveal an early granulomatous response consisting of macrophages, eosinophils, fibroblasts and plasma cells [Figure 6.2.5 *(c)-(h)*]. From 4 wpt, worm internal structures were compromised [Figure 6.2.5 *(b)*] and as the parasites were resorbed, the nodule area reduced markedly, leading to complete resolution of some nodules by 48 wpt.

Figure 6.2.1. Complete cross-section of untreated onchocercoma. Image stitched from 4 quadrant sections photographed at 2× magnification. Worm sections (black arrow heads) are situated within a granulocytic cellular environment (circle); the nodule is enclosed within a thick fibrous capsule (black arrow). Scale bar 250 µm. Giemsa stain.

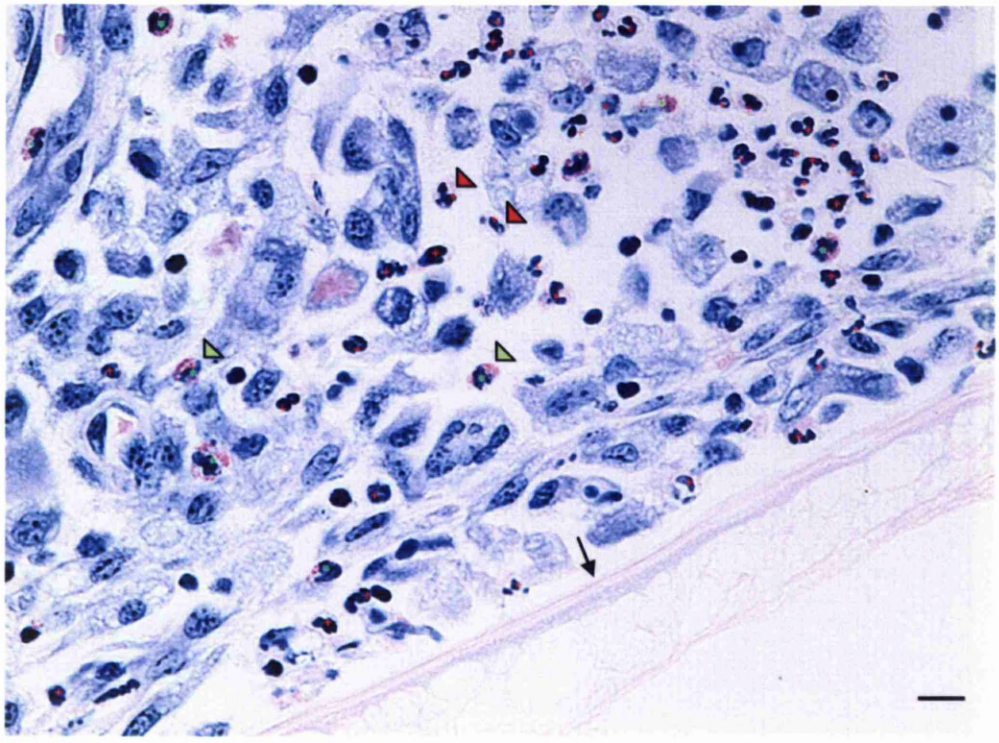
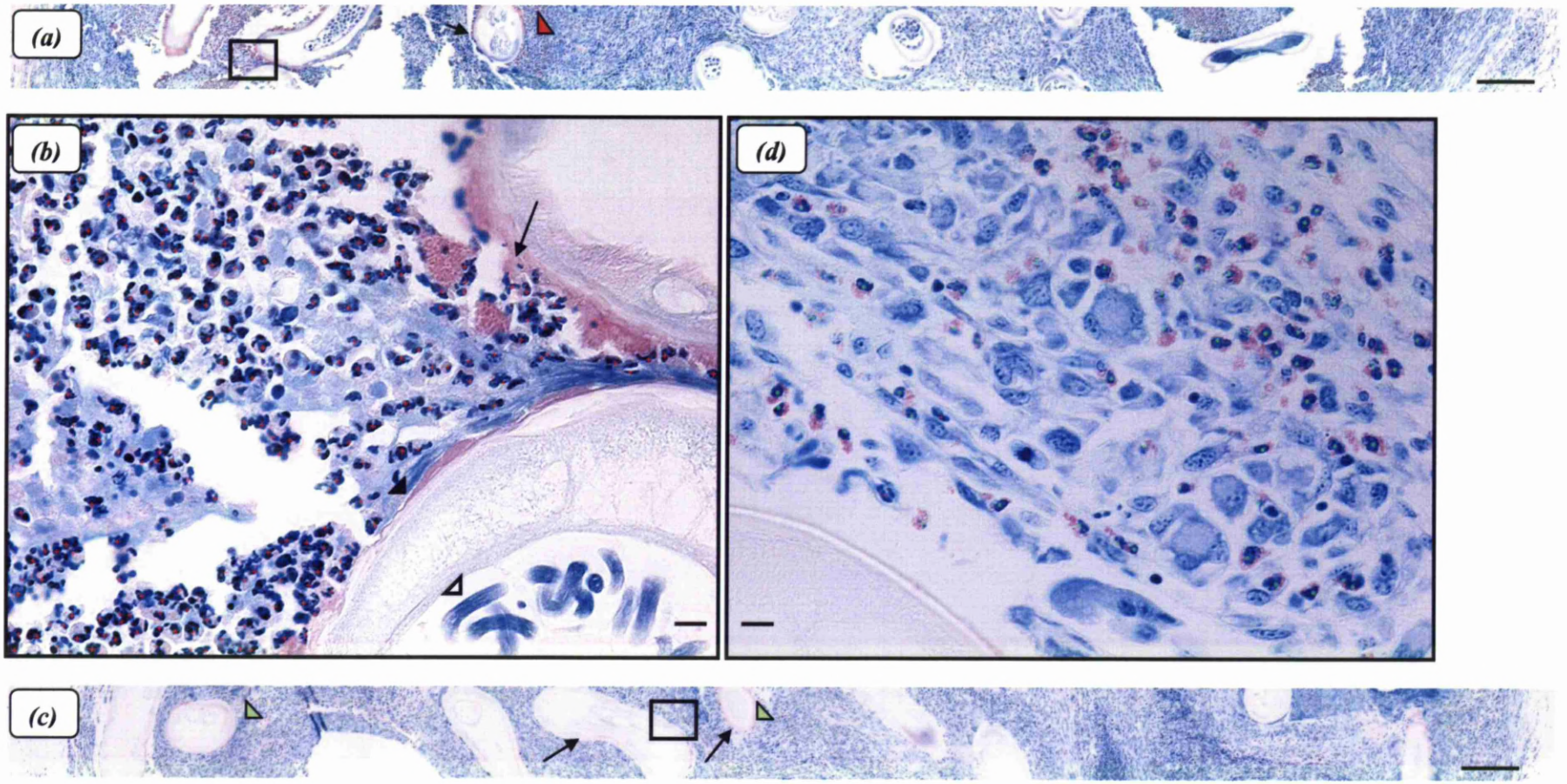


Figure 6.2.2. Mixed granulocyte infiltrate adjacent to worm cuticle (arrow) within a tetracycline-treated nodule extirpated at 8 wpt. Image annotated to quantify neutrophils (red arrowheads) and eosinophils (green arrowheads), which were digitally marked with red crosses or green stars, respectively. Giemsa stain. Scale bar 10 μm.

Figure 6.2.3. (following page). Annotated images to illustrate differences in cell populations within nodules from untreated controls [(a) and (b)] and tetracycline-treated animals [(c) and (d)]. (a) Transect stitched from images captured at 20× magnification. Areas of red crosses (red arrowhead) illustrate neutrophilic infiltrate around healthy worms (black arrow). Open box indicates enlarged area [(b)]. Scale bar 250 μm. (b) Red crosses mark neutrophils adjacent to cuticles of healthy female worm (closed arrowhead) containing microfilariae (open arrowhead); or intermingled with Splendore-Hoeppli deposits (black arrow) Scale bar: 10 μm. (c) Stitched transect from tetracycline-treated nodule extirpated 12 wpt. Areas of green stars illustrate eosinophilic infiltrate (green arrowhead) around worms devoid of visible reproductive contents (arrows) Open box indicates enlarged area [(d)]. Scale bar 250 μm (d) Green stars mark eosinophils which have infiltrated into the treated nodule. Note lack of neutrophils. Scale bar: 10 μm. Stain: Giemsa.


(a)
(b)
(d)
(c)

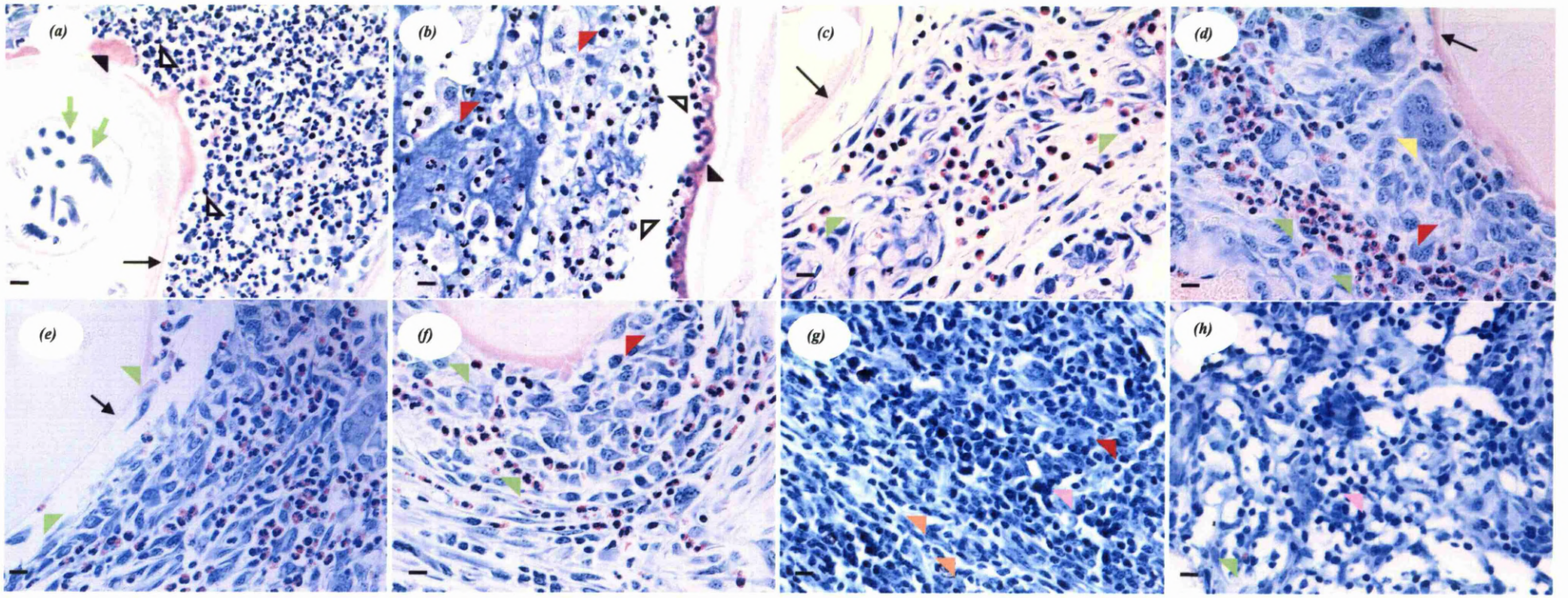

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)

Figure 6.2.4. (previous page) Histopathology of *O. ochengi* onchocercomata from tetracycline treated animals. *(a)* Nodule removed at 2 wpt displaying intense neutrophilic infiltrate (open arrowheads) in close proximity to the worm cuticle (black arrow) and Splendore-Hoeppli (SH) deposits (closed arrowhead). Microfilariae are present in the uterus (green arrows). *(b)* Section from 4 wpt nodule, showing a highly neutrophilic infiltrate (open arrows) close to and in contact with the Splendore-Hoeppli deposits (SH) (closed arrowhead). Macrophages are also present (red arrowhead). *(c)* Section from 8 wpt. Eosinophils are the most abundant granulocyte (green arrowhead) and are adjacent to the worm cuticle (black arrow). Note lack of neutrophils from this time point onwards. *(d)* Section from 12 wpt. A 'band' of eosinophils (green arrowheads) is present parallel to the worm cuticle (black arrow). Macrophages (red arrowhead) and giant cells (yellow arrowhead) are also present. *(e)* Section from 24 wpt. Eosinophilic infiltrate remains and some (green arrowheads) are attached to the worm cuticle (black arrow). Note the altered internal structure in the worm section. *(f)* Section from 36 wpt. Eosinophils (green arrowheads) are seen in approximately equal numbers with macrophages (red arrowheads). *(g)* and *(h)* Sections from 48 and 54 wpt respectively, showing a granuloma-type appearance, characterised by lymphocytes (pink arrowhead), fibroblasts (orange arrowhead), macrophages (red arrowhead) and an eosinophil (green arrowhead). Note lack of worm sections as resolution had occurred. Stain: Giemsa. Scale bars: 10 μm.

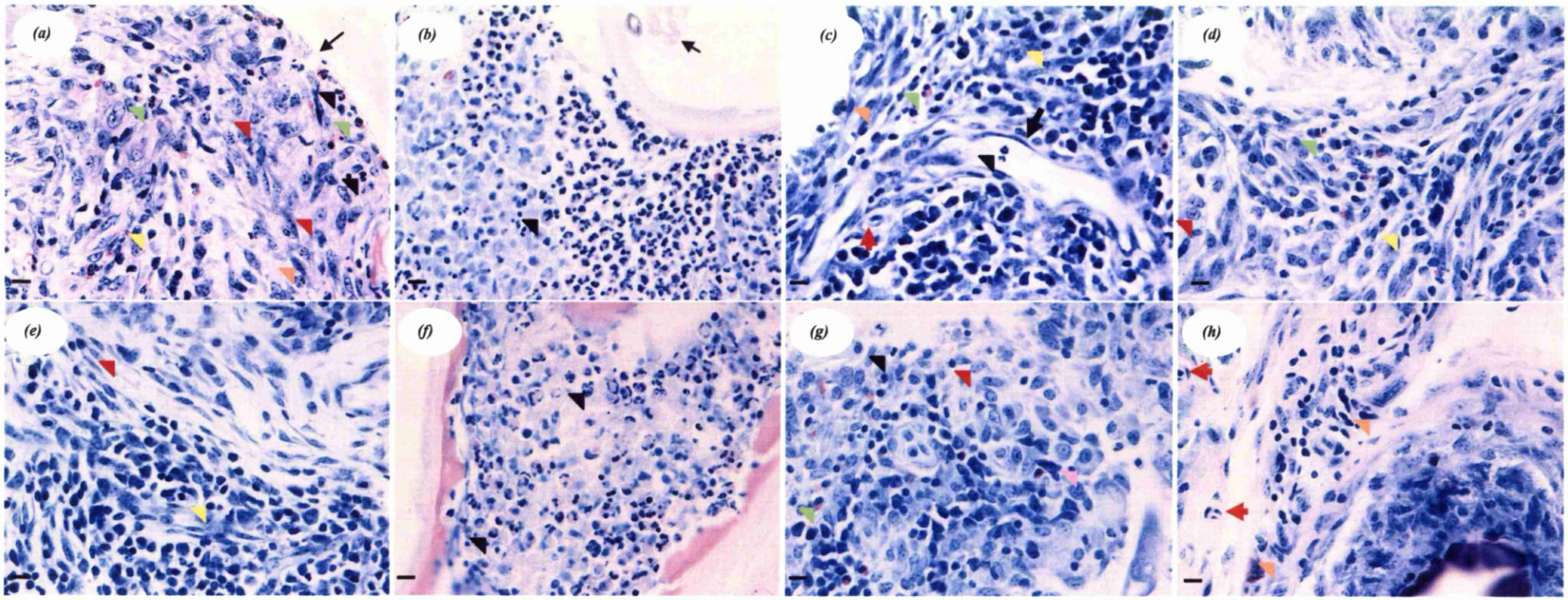


Figure 6.2.5. Nodules removed from animals receiving melarsomine chemotherapy. Macrophages (red arrowheads), eosinophils (green arrowheads), neutrophils (black arrowheads) lymphocytes (yellow arrowheads), fibroblasts (orange arrowheads), plasma cells (pink arrowheads). *(a)* Section at 2 wpt shows mixed cellular infiltrate in the immediate vicinity of the worm cuticle (black arrow). *(b)* Section at 4 wpt. Rich neutrophilic infiltrate surrounds the worm which contains a section of convoluted uterus (black arrow). *(c)* Section at 8 wpt. Lymphocytic infiltrate with few eosinophils (green arrowheads), neutrophils and fibroblasts. Longitudinal (black arrow) and transverse (red arrow) sections of blood vessels are present. *(d, (e, (f), (g), (h)* Sections at 12, 24, 36, 48 and 54 wpt respectively. Note lack of worm sections within most of the resolving nodules. Stain: Giemsa. Scale bar: 10 μm

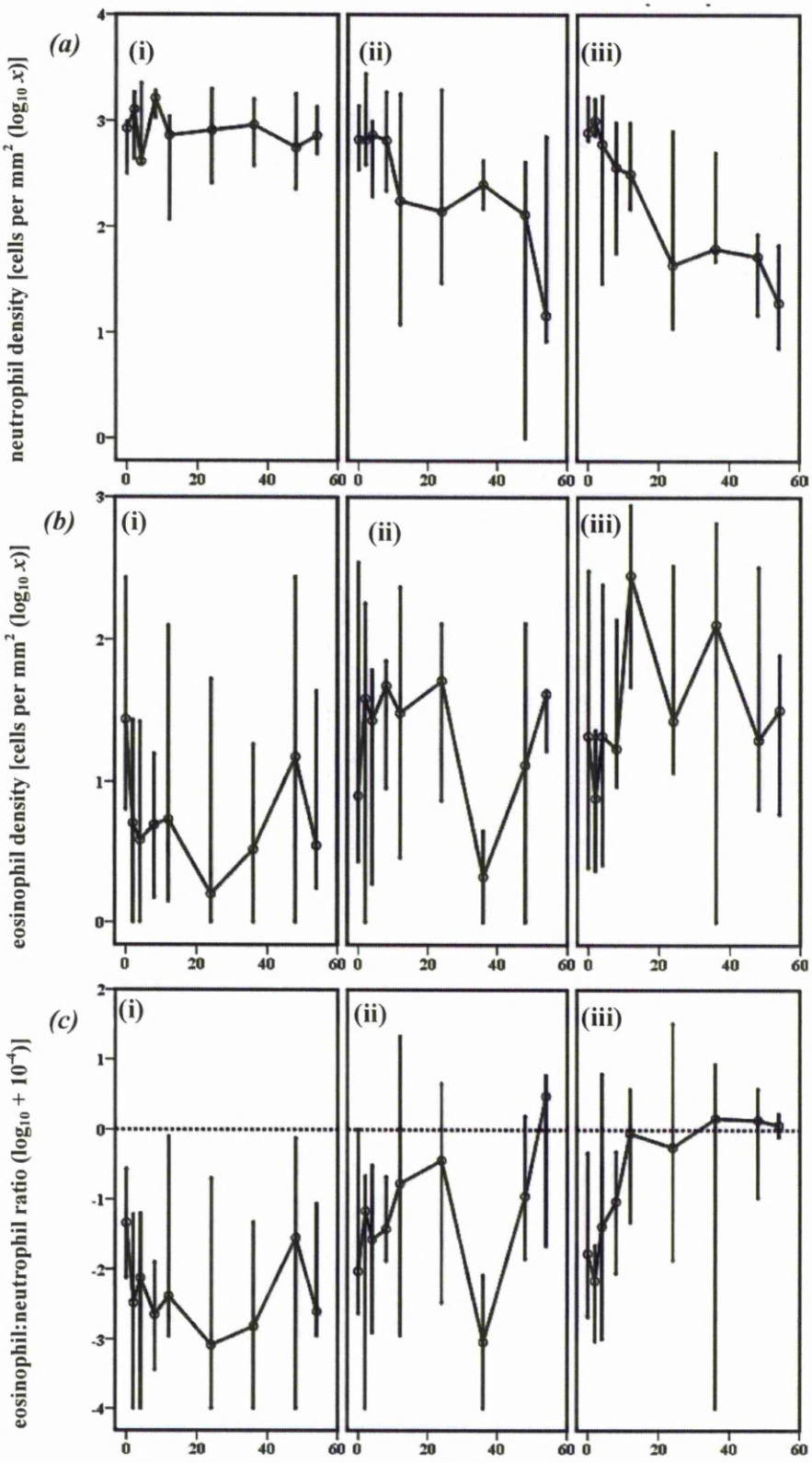


Figure 6.2.6. Granulocyte quantification within *O. ochengi* onchocercomata following [a(iii), b(iii), c(iii)] oxytetracycline or [a(ii), b(ii), c(ii)] melarsomine chemotherapy, or [a(i), b(i), c(i)] in the absence of treatment. (a) Transect neutrophil density (median and range). (b) Transect eosinophil density (median and range). (c) Eosinophil:neutrophil ratios (median and range) The horizontal dotted line represents parity between the two cell types. $n = 5$ (i;ii) or 6 (iii).

6.3. Discussion

6.3.1. Comparison of the cellular infiltrate in *Onchocerca* spp. chemotherapeutically treated to deplete *Wolbachia*

This section of the current study was designed to further investigate findings from an experiment conducted by Nfon et al (2006), who performed complete quantitative cytological analyses of oxytetracycline-treated *O. ochengi* nodules. Of the cell types evaluated, only the granulocyte populations fluctuated in response to treatment, and although other cells (e.g. macrophages, mast cells) were frequently present in treated nodules, numbers either remained constant (as with macrophages) or varied with no distinct patterns evident (Nfon et al., 2006). This information provided the background for the specific quantification of granulocytes within our longitudinal study.

The results from the oxytetracycline group in our investigation strongly support those reported from the study of Nfon et al. (2006), where the decrease of neutrophil numbers was linked to a depletion of *Wolbachia*. The Nfon et al. (2006) study showed that in the SIR and COM groups, eosinophils infiltrated into the nodule by 8 wpt, and in some sections had outnumbered neutrophils by 12 wpt. At 48 weeks in the SIR group, neutrophils resumed predominance within the central zones of nodules; however in the COM group, neutrophils continued to be abated and the nodule composition was described as a wound-healing milieu, consisting of eosinophils, macrophages, fibroblasts, giant cells, plasma cells and lymphocytes. The influx of eosinophils was found to correlate with an earlier report that identified the virtual elimination of *Wolbachia* at 8 wpt in both COM and SIR groups; and subsequent recrudescence by 24 wpt in the SIR group only (Gilbert et al., 2005). Relatively late infiltration of eosinophils in the melarsomine group during our

experiment indicates that these cells participate in the clearance of worm fragments and nodule resolution, in conjunction with other cells that are active during a typical wound healing response. In contrast, the much earlier recruitment of eosinophils in the oxytetracycline-treated group, suggests that these cells were attracted by a distinct mechanism as primary effector cells, as opposed to a component of a secondary response following worm death.

The highly neutrophilic infiltrate observed forming layers around filariae in *O. ochengi* control nodules in this investigation accords with previous descriptions of untreated *O. ochengi* (Wildenburg et al., 1997) and *O. volvulus* nodules (Brattig et al., 2001). These authors also observed that some worms reside in cysts with few inflammatory cells present, while others were completely surrounded by multiple layers of neutrophils, with fewer macrophages and giant cells also present. In *O. volvulus*, the cysts surrounding male worms and the anterior ends of female worms were found to contain defensins, and thus were attributed to the neutrophilic environment. Furthermore, the cysts were hypothesised to contribute to the uptake of soluble nutritional factors by the worm and possibly to facilitate mating (Brattig et al., 2001). In our study, neutrophils were frequently present in close proximity to worms in control nodules, yet there were also areas around some worms that contained no cells at all. These areas could be cysts, but artefacts from nodule processing could not be ruled out, as the sections were not specifically stained for cell secretions.

6.3.2. Comparison of the cellular infiltrate around naturally *Wolbachia*-positive and -negative *Onchocerca* spp.

Wolbachia-depleted *O. ochengi* nodules in this study showed a similar cellular composition to the aposymbiotic *O. flexuosa* (Brattig et al., 2001), which in their

natural state are densely infiltrated by eosinophils in the centre of the nodule (Wildenburg et al., 1997) and additionally in the capsule. In *O. flexuosa* nodules, neutrophils were absent (Brattig et al., 2001). This is a stark contrast to other nodule-forming non-motile filarial worms that regularly exhibit layers of neutrophils around living worms, such as *O. volvulus* in man (Brattig et al., 2001), *O. gibsoni* in cattle (Wildenburg et al., 1997) and *O. jakutensis* in red deer. Moreover, nodules from deer co-infected with *O. jakutensis* and *O. flexuosa* nodules exhibited the same cellular differences, which effectively rules out host differences (Brattig et al., 2001).

Interestingly, the *Wolbachia*- replete (Neary et al., 2010) nodule-forming bovine *Onchocerca armillata* does not share a similar granulocytic nodular environment. In fact, many viable *O. armillata* worms have no surrounding inflammatory response at all (Neary et al., 2010). In some nodules, macrophages were seen close to the cuticle of dead and degenerating worms, with sparse neutrophils and eosinophils (except in older nodules where an increased prevalence of eosinophils was recorded). These remained peripheral, and granulocytes were never observed adhering to or degranulating on the worm cuticle (Neary et al., 2010). In addition, young *O. armillata* nodules contain an exudate, which is caseous in older nodules, and they are also commonly calcified (Atta el Mannan et al., 1984; Neary et al., 2010). In contrast, although old *O. ochengi* nodules from non-treated cattle are sometimes calcified as was seen in the current experiment, calcification was not associated with chronicity within treated nodules; rather these nodules became progressively smaller, before resolving.

6.3.3. Evidence for a parallel scenario within *O. volvulus*

Longitudinal studies in humans are extremely limited due to reasons of ethics, compliance and cost. There are no publications to our knowledge that describe the cellular dynamics of *O. volvulus* nodules from the same patients over time (which highlights the importance of the *O. ochengi* model for long-term studies). However, snapshots of nodules removed from untreated *O. volvulus* patients were similarly found to display a strong neutrophilic infiltrate adjacent to live adult worms; in contrast to depleted neutrophil numbers in patients treated with 6 weeks of daily doxycycline, in which worm sections at 4 and 9 months after treatment were almost completely devoid of *Wolbachia* by immunohistochemistry [IHC (Brattig et al., 2001)]. Nodules treated with doxycycline contained far fewer neutrophils adjacent to live worms, but neutrophils were evident together with macrophages and giant cells in the vicinity of worms that had died naturally (Brattig et al., 2001).

The phenotypic comparison of *O. volvulus* and *O. ochengi* suggest that the females of both species are sessile (Bain, 1981; Franz et al., 1987). Both species contain *Wolbachia* in their lateral hypodermal cords, and both attract a neutrophilic infiltrate in their natural states, which can be abrogated by tetracycline chemotherapy.

The results presented here, together with the data from other studies, illustrate that these viable, non-motile, nodule-forming *Onchocerca* spp. which contain *Wolbachia* are surrounded by neutrophils, and that the neutrophilic milieu decreases in synchrony with chemotherapy directed against *Wolbachia*. Furthermore, eosinophils are normally scarce and remain at the outer edge of the neutrophilic infiltrate. However, eosinophil numbers increase relatively early in nodules specifically depleted of *Wolbachia*, in contrast to the relatively late and statistically non-

significant eosinophilia within nodules that were treated with a macrofilaricidal drug that does not exert a direct anti-*Wolbachia* effect.

7. The effect of oxytetracycline treatment of cattle on the expression of neutrophil and eosinophil-associated cytokines in *Onchocerca ochengi* nodules.

7.1. Introduction

Selected bovine transcripts were quantified using qRT-PCR from nodules removed during the longitudinal experiment, to determine the expression of granulocyte-related cytokines within nodules treated with oxytetracycline or melarsomine, compared with untreated controls.

The cytokine profile of *O. ochengi* nodules has not previously been reported; thus, the selection of cytokine targets was based on the limited data available for cytokines identified in *O. volvulus* nodules and in other animal models or *in-vitro* systems. In a groundbreaking study conducted by Brattig et al. (2001), untreated *O. volvulus* extracts were shown to induce greater amounts of TNF-α from human blood monocytes and macrophages compared to cells cultured with *Wolbachia*-free extracts, which only produced low levels of TNF-α. Similarly, IL-8 production by monocytes and macrophages when incubated with untreated *O. volvulus* was increased by a maximum of 7-fold, compared to *Wolbachia*-depleted worms, and a comparable trend was reported in response to incubation with *O. ochengi*, in contrast to the *Wolbachia*-negative *A. viteae* (Brattig et al., 2001). Further *in-vitro* assays showed that recombinant WSP from *D. immitis* stimulated the production of IL-8 from canine neutrophils (Bazzocchi et al., 2003). However, as these assays were performed *in-vitro*, their relevance to filarial infection *in vivo* has yet to be established.

Regarding the role of eosinophilic cytokines, eotaxin was shown to facilitate eosinophil recruitment into the skin of DEC-treated patients *ex-vivo* (Pearlman et al., 1999), and into the peritoneal cavity of *B. malayi* infected mice *in-vivo* (Simons et al., 2005). Also in the *B. malayi* rodent model, IL-5 was shown to be instrumental in the differentiation of eosinophils and release into the circulation, whereas eotaxin was necessary for the recruitment of eosinophils into the tissues (Simons et al., 2005). In addition, IL-5 was responsible for eosinophilia following injection of *O. lienalis* microfilariae in mice (Folkard et al., 1996).

Building upon our previous data revealing the cellular dynamics involving neutrophils and eosinophils in treated nodules, selected cytokine transcripts were measured using absolute quantification by qRT-PCR during the period of major granulocyte population shifts; *i.e.*, between 8 and 24 wpt (Section 6.2.1). Thus, the neutrophilic cytokines IL-8 and the GRO protein family, together with the eosinophilic cytokines IL-5 and eotaxin, were quantified and normalised against the bovine housekeeping gene 28S rRNA.

7.2. Results

7.2.1. Effect of treatment on the neutrophilic cytokines

The IL-8:28S rRNA ratio was significantly lower in nodules from oxytetracycline-treated animals compared with those from the control group (

Figure 7.2.1 *(a)*; GLM repeated measures, Tukey's post-hoc test: mean difference = -1.0 log, $p = 0.010$). This was in contrast to nodules from the melarsomine-treated group, where the ratio was not significantly different from that of the controls (Tukey's post-hoc test: mean difference = -0.7 log, $p = 0.107$). The same trend was

observed with the GRO:28S rRNA ratio, which was also significantly reduced in nodules from the oxytetracycline-treated group compared to control animals (

Figure 7.2.1 *(b)*; GLM repeated measures, Tukey's post-hoc test: mean difference = -0.7 log, $p = 0.044$),whereas this was not the case in the melarsomine-treated group (Tukey's post-hoc test: mean difference = -0.4 log, $p = 0.296$).

7.2.2. Effect of treatment on the eosinophilic cytokines

In contrast to the neutrophilic cytokines, there were no overall significant changes in IL-5 [Figure 7.2.2 *(a)*] or eotaxin [Figure 7.2.2 *(b)*] expression between 8 and 24 wpt in either of the treatment groups when compared to the controls. For the IL-5:28S rRNA ratio, the mean difference between the oxytetracycline and the control groups was 0.03 log (GLM repeated measures, Tukey's post-hoc test; $p = 0.991$), and for the melarsomine and the control groups it was 0.09 log ($p = 0.918$). For the eotaxin:28S rRNA ratio, the mean difference between the oxytetracycline and the control groups was -0.3 log (GLM repeated measures, Tukey's post-hoc test; $p = 0.814$), and for the melarsomine and the control groups it was -0.5 log ($p = 0.641$).

The IL-8:IL-5 ratio was significantly reduced within nodules from both treatment groups compared to the control group (Figure 7.2.3). For oxytetracycline-treated nodules, the mean difference was -1.0 log (GLM repeated measures, Tukey's post-hoc test; $p = 0.005$), and for the melarsomine-treated group it was -0.7 log, ($p = 0.034$). Furthermore, the interaction between the IL-8:IL-5 ratio and time was also significant (GLM repeated measures, $p = 0.004$).

7.2.3. Relationship between bovine cytokines and *Wolbachia*

There were statistically significant, modest or strong positive correlations between bovine IL-8 and *Wolbachia* copy numbers (Figure 7.2.4) throughout all three time points. The Pearson product-moment correlation coefficient was $r = 0.624$ ($p = 0.010$) for 8 wpt [Figure 7.2.4 *(a)*]; $r = 0.572$ ($p = 0.020$) for 12 wpt [Figure 7.2.4 *(b)*]; and $r = 0.708$ ($p = 0.002$) for 24 wpt [Figure 7.2.4 *(c)*]. A similar pattern was evident for bovine GRO, which exhibited statistically significant, modest correlations when plotted against *Wolbachia* (Figure 7.2.5) at all time points except 12 wpt (which showed a positive, albeit non-significant, trend). The Pearson product-moment correlation coefficients were: $r = 0.625$ ($p = 0.010$) for 8 wpt [Figure 7.2.5 *(a)*]; $r = 0.452$ ($p = 0.070$) for 12 wpt [Figure 7.2.5 *(b)*]; and $r = 0.699$ ($p = 0.003$) for 24 wpt [Figure 7.2.5*(c)*].

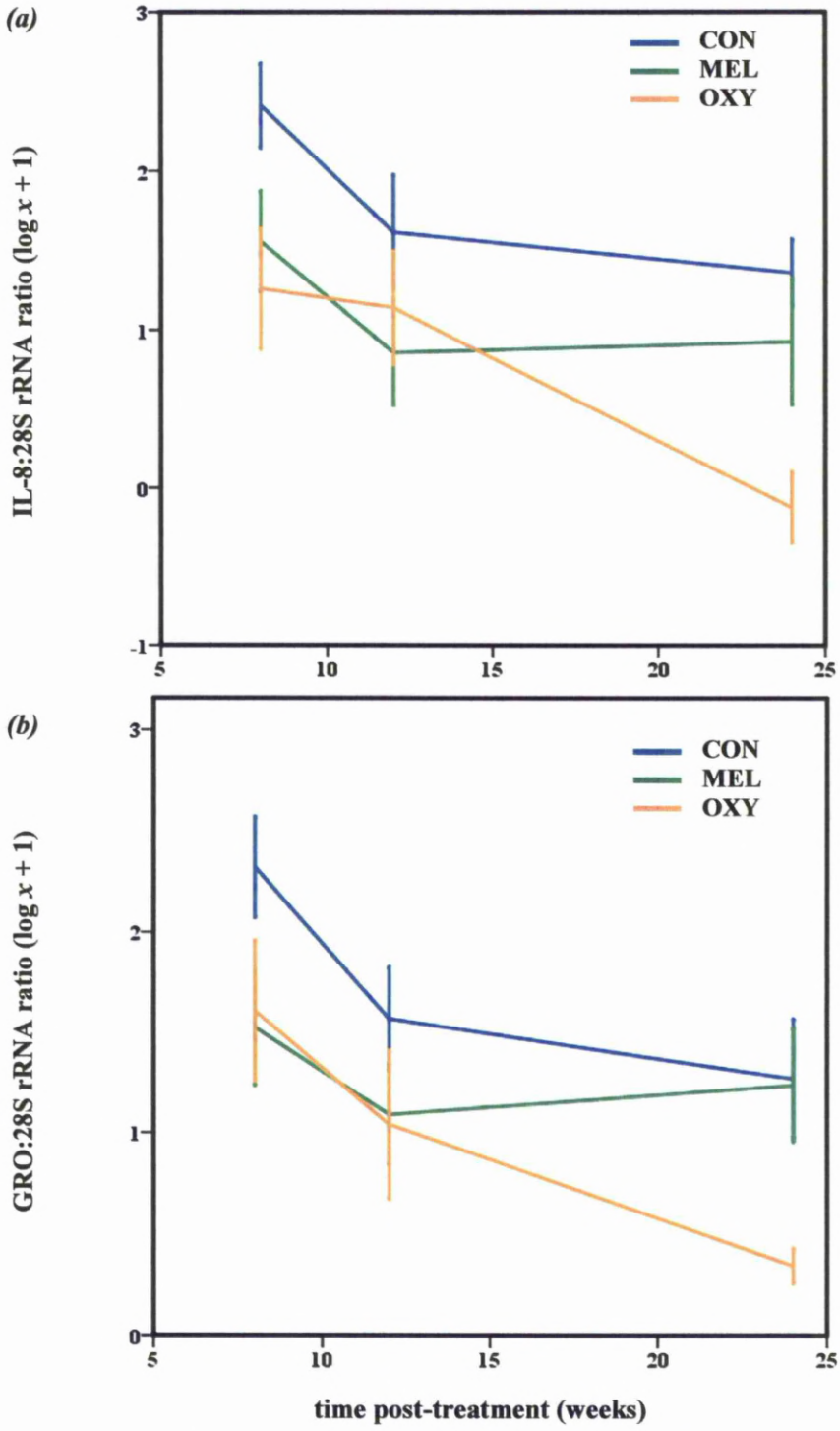


Figure 7.2.1. Quantification of bovine transcripts (normalised against 28S rRNA) for the neutrophilic chemokines *(a)* interleukin-8 (IL-8) and *(b)* the growth-regulated (GRO) protein family in onchocercomata treated with oxytetracycline ($n = 6$) or melarsomine ($n = 5$), or in the absence of treatment ($n = 5$). Lines represent the mean ± 1 SE.

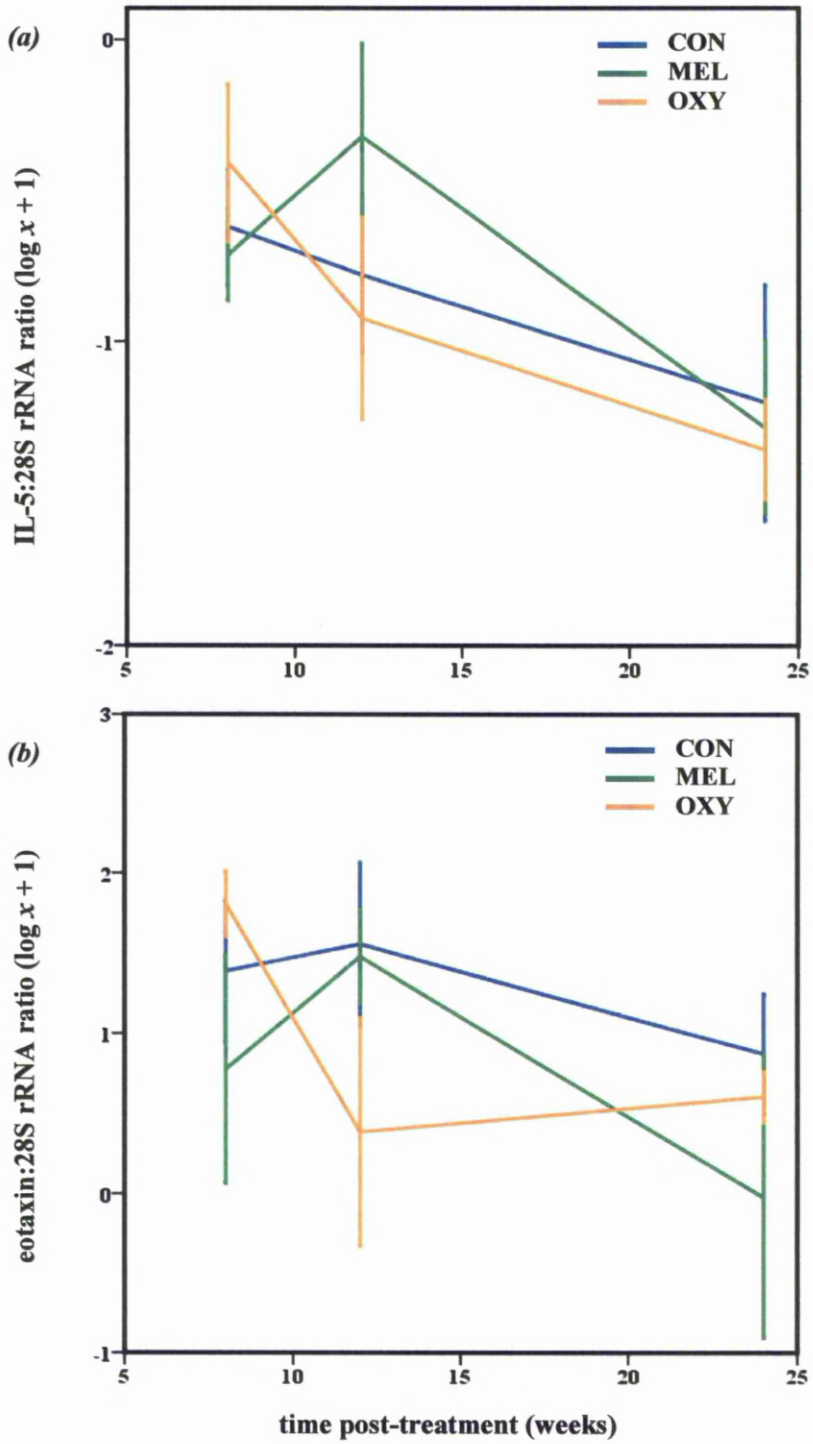


Figure 7.2.2. Quantification of bovine transcripts (normalised against 28S rRNA) for the eosinophilic cytokines *(a)* interleukin-5 (IL-5) and *(b)* eotaxin in onchocercomata treated with oxytetracycline (n = 6) or melarsomine (n = 5), or in the absence of treatment (n = 5). Lines represent the mean ± 1 SE.

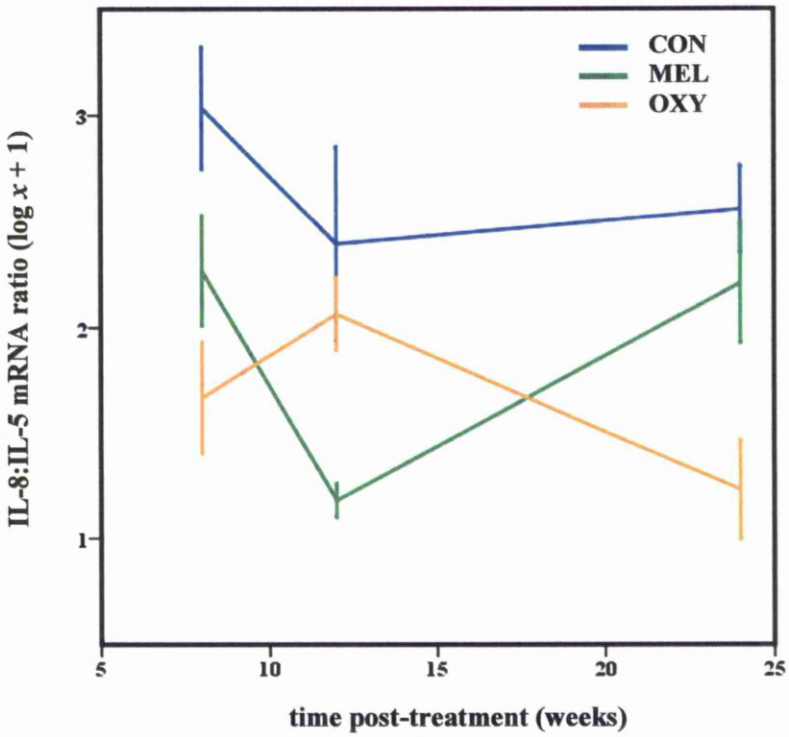


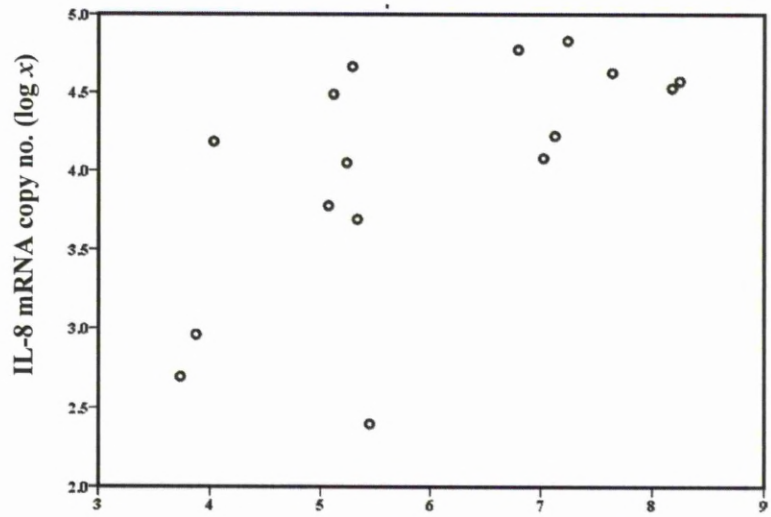
Figure 7.2.3. Bovine IL-8:IL-5 mRNA ratios from onchocercomata treated with oxytetracycline (n = 6) or melarsomine (n = 5), or in the absence of treatment (n = 5). Lines represent the mean ± 1 SE.

Following Pages

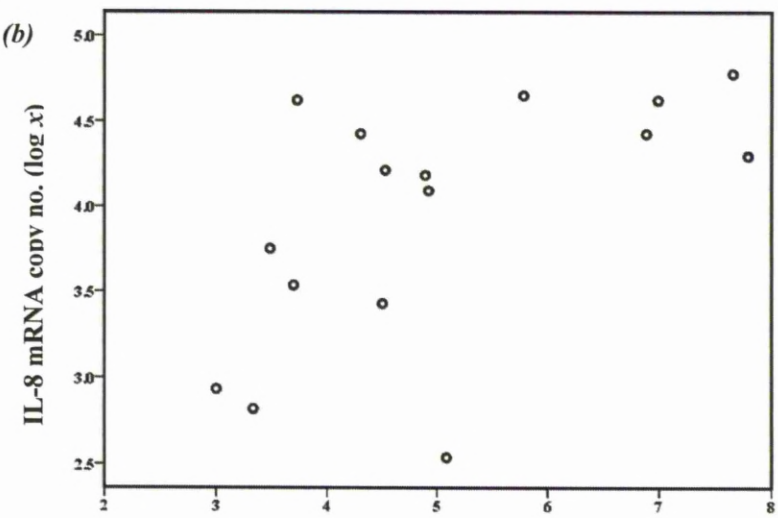
Figure 7.2.4. Scatter-plot of bovine IL-8 mRNA against *Wolbachia* 16S rRNA transcripts from onchocercomata removed *(a)* 8 wpt, *(b)* 12 wpt and *(c)* 24 wpt. Points represent data for individual animals from all three experimental groups

Figure 7.2.5. Scatter-plot of bovine GRO mRNA against *Wolbachia* 16S rRNA transcripts from onchocercomata removed *(a)* 8 wpt, *(b)* 12 wpt and *(c)* 24 wpt. Points represent data for individual animals from all three experimental groups.

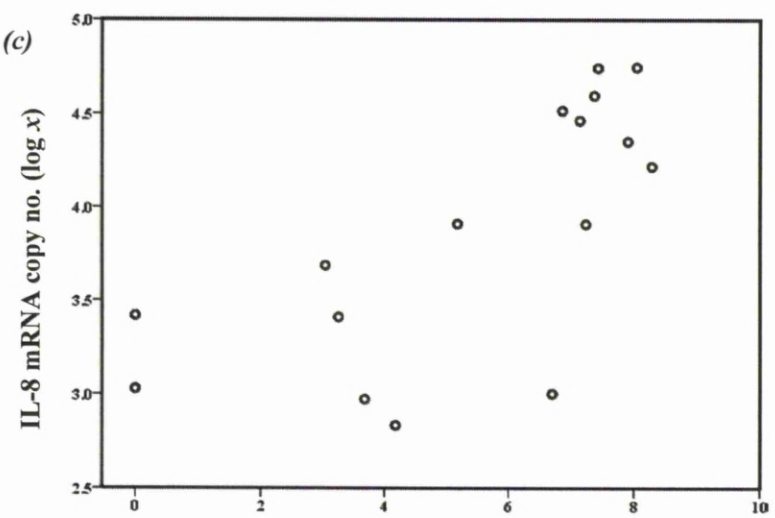
7.3.4 ***(a)***



(b)

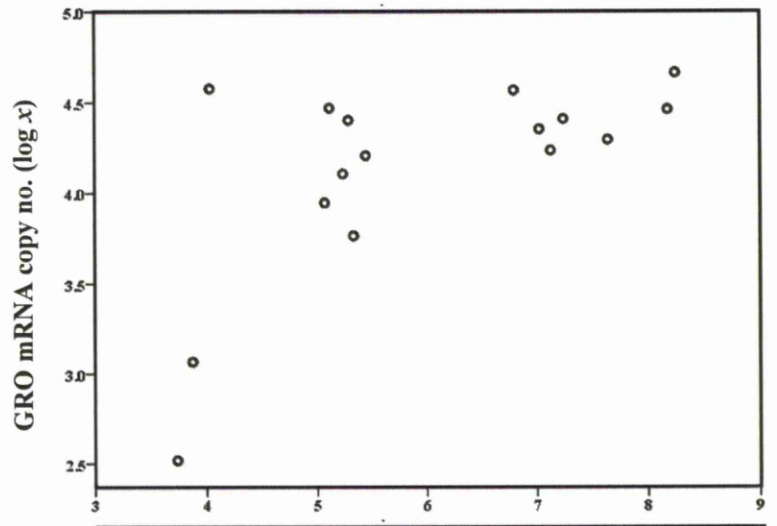


(c)

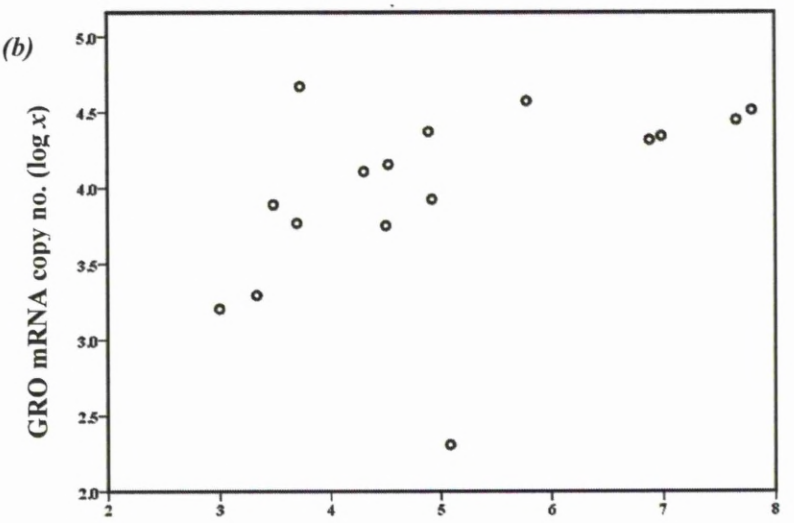


***Wolbachia* 16S rRNA copy no. (log x + 1)**

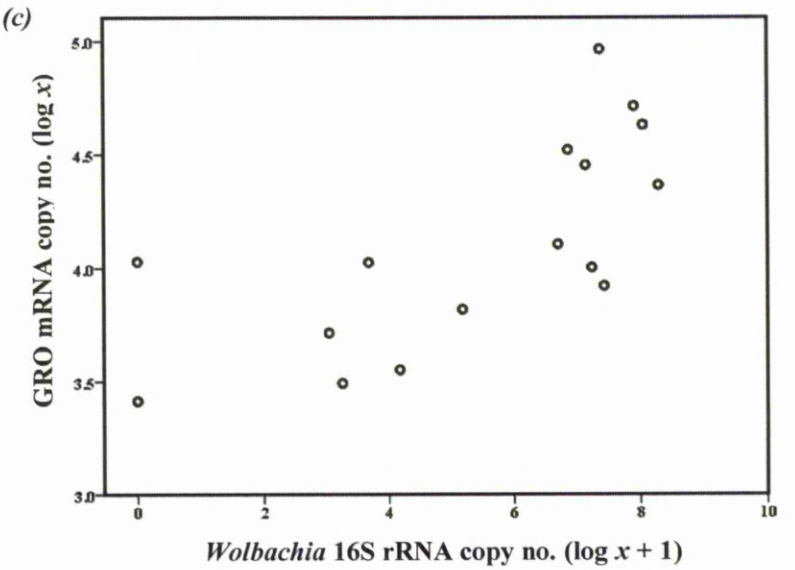
7.3.5 ***(a)***


GRO mRNA copy no. (log x)
5.0
4.5
4.0
3.5
3.0
2.5
3
4
5
6
7
8
9

(b)


GRO mRNA copy no. (log x)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
2
3
4
5
6
7
8

(c)


GRO mRNA copy no. (log x)
5.0
4.5
4.0
3.5
3.0
0
2
4
6
8
10
Wolbachia 16S rRNA copy no. (log x + 1)

7.3. Discussion

7.3.1. The role of neutrophilic cytokines and their relationship with *Wolbachia*

The fact that IL-8 and GRO transcripts were significantly reduced in the oxytetracycline group, with no significant change in the melarsomine group, suggests that *Wolbachia* may be involved in their induction. In addition, the statistically significant, positive correlation between the neutrophilic cytokines and *Wolbachia* evident in all the experimental groups is compatible with this interpretation. Treatment groups were combined for the analysis to increase the sample size, as the alternative approach of combining data from different time-points would have violated independence by using several observations from each individual animal. Indeed, the conservation of the positive correlation between IL-8 or GRO and *Wolbachia* was to be expected across all experimental groups if a dependent relationship was the case. It could be argued that confounding factors such as nodule size may have influenced the correlation; however, there was no significant correlation between *Wolbachia* 16S and bovine 28S rRNA copy numbers (data not shown), indicating that the number of mammalian cells in the nodule and *Wolbachia* levels were not conflated. In addition, WSP from *D. immitis* was demonstrated to induce IL-8 production in canine neutrophils (Bazzocchi et al., 2003), and isolated *Wolbachia* and *Wolbachia*-containing *B. malayi* extracts stimulated direct production of TNF-α, macrophage inflammatory protein 2 and the murine GRO orthologue, keratinocyte-derived cytokine (KC) by murine peritoneal neutrophils *in-vitro* (Gillette-Ferguson et al., 2004). Furthermore, following instillation of untreated *O. volvulus* worm extract into the murine cornea, *Wolbachia* induced the production of CXC chemokines by resident cells via TLR-2 activation, thus facilitating the recruitment of neutrophils (Gillette-Ferguson et al., 2007). These

data support the suggestion that a similar mechanism could operate in the *O. ochengi* nodule.

The significant decrease in IL-8 and GRO in oxytetracycline-treated nodules appeared to succeed, rather than precede, the reduction in neutrophils that was observed via histopathology in the oxytetracycline group from 8 wpt (section 6.2.1), as no marked reduction in chemokine levels was seen until 24 wpt. While other studies have shown that bovine IL-8 attracts and activates bovine neutrophils *in-vitro* (Caswell et al., 1999) and *in-vivo* (Mitchell et al., 2003), it is also the case that neutrophils can produce IL-8 (Bazzoni et al., 1991b) and GRO (Ryu et al., 2004) to perpetuate further neutrophilic activation and recruitment. Thus, although we have revealed a specific effect of oxytetracycline treatment on neutrophilic chemokine levels, we did not attempt to determine the cellular origin of IL-8 and GRO production, and nor could we distinguish whether their decline is a cause or effect of the decrease in neutrophil numbers.

7.3.2. The role of eosinophilic cytokines and their relationship with *Wolbachia*

Although eotaxin has been identified as an important chemoattractant in dermal (Pearlman et al., 1999) and ocular (Simons et al., 2005) manifestations of onchocerciasis, and is known to attract bovine eosinophils *in-vitro* (Vogel et al., 2005), it was not found to be influential in the recruitment of eosinophils into the oxytetracycline-treated *O. ochengi* nodule in our study. Similarly, IL-5 did not appear to play a role in the recruitment of eosinophils into the antibiotic-treated nodules, which was evident from 12 wpt from the histopathology data (section 6.2.1). The absence of any change in eotaxin levels is particularly surprising because in filarial models, eotaxin appears to be associated with the recruitment of

eosinophils into the tissues (Pearlman et al., 1999; Simons et al., 2005) , whereas IL-5 drives the initial development of peripheral eosinophilia (Folkard et al., 1996; Limaye et al., 1991). However, eotaxin can also stimulate the release of eosinophils into the circulatory pool (Palframan et al., 1998), and both cytokines have been shown to synergise *in-vivo* to facilitate eosinophil accumulation (Collins et al., 1995). The intrinsic limitations of qRT-PCR mean that cytokine upregulation can only be evaluated at the mRNA level. This method excludes the quantification of cytokines present at the protein level released from preformed granules within mast cells or eosinophils already present in the nodule; an omission that could be overcome by performing immunocytochemistry for cytokine targets as described by Persson et al (2003). Furthermore, bursts of cytokine expression may have been overlooked, due to the relatively long intervals between time-points, compared to the relatively short half-life of active cytokines (Hillyer and Male 2005).

7.3.3. Consideration of other potentially relevant cytokines

The neutrophilic chemokine ENA-78 was produced by mast cells and induced the migration of neutrophils into the airways of mice in a model of allergic airway inflammation (Lukacs et al., 1998). In the bovine, an ENA-78 homologue was secreted by alveolar epithelial cells in inflamed lung tissues, and was found to induce migration and activation of bovine neutrophils, causing shape change and aggregation (Allmann-Iselin et al., 1994). It has also been demonstrated that human eosinophils express ENA-78, which may recruit neutrophils to sites of inflammation (Persson et al., 2003). As ENA-78 has not been characterised in onchocercomata to date, it constitutes an interesting candidate for further studies of nodule cytokine expression.

The mechanisms for the recruitment of eosinophils into the antibiotic-treated nodule deserve further elucidation. Other eosinophilic cytokines that could be instrumental in the recruitment of eosinophils include RANTES and IL-33. RANTES (together with eotaxin) was expressed by the dermal epithelium of onchocerciasis patients after ivermectin treatment, and was associated with eosinophil recruitment (Cooper et al., 2000). Furthermore, the increased expression of bovine RANTES from ovarian macrophages around the post-ovulatory period coincided with an influx of eosinophils into the bovine ovary (Aust et al., 1999). Although not primarily chemotactic, IL-33 has been shown to activate and extend the life of eosinophils *in-vitro* (Cherry et al., 2008), and can indirectly recruit eosinophils via the induction of eotaxin release (Matsuba-Kitamura et al., 2010). Homologues of both RANTES and IL-33 have been identified in the bovine genome (Zimin et al., 2009), and would be appropriate targets to further characterise the mechanisms of eosinophil recruitment in the *O. ochengi* nodule.

8. Effect of oxytetracycline treatment of cattle on eosinophil degranulation in *Onchocerca ochengi* nodules.

8.1. Introduction

To compare the activation of eosinophils between treated animals and non-treated controls, semi-quantification of degranulating eosinophils was performed on nodule transects. In addition, to relate worm viability and reproductive status with the presence of activated eosinophils in treated or untreated animals, adjacent sections from *ex-vivo* nodules were processed to identify *Wolbachia* using IHC (targeting WSP), followed by Giemsa counterstain for the semi-quantitative analysis of eosinophil clusters.

Previously, the involvement of eosinophils in the active destruction of *O. ochengi* after *Wolbachia* depletion could only be inferred (Nfon et al., 2006). However, the design of our study enabled the semi-quantification of degranulating eosinophils concomitantly with the quantification of neutrophil and eosinophil populations (Section 3.2). Our data revealed that the cellular changes in the nodule were most pronounced between 8 and 24 wpt (Chapter 6), and these time-points were selected for a detailed study of clusters of eosinophils in association with specific worm features in adjacent nodule sections.

8.2. Results

8.2.1. Effect of treatment on eosinophil degranulation

An analysis of degranulating eosinophils on *ex-vivo* nodule transects, performed using a semi-quantitative scoring system, revealed a marked difference between

melarsomine-treated and oxytetracycline-treated worms (Figure 8.2.1). In both melarsomine and control groups, the median degranulation score never exceeded 1 (<1 degranulating eosinophil per worm section). Furthermore, there was no significant change over time in either the melarsomine-treated group (Friedman test: $\chi^2 = 4.9$, $p = 0.605$) or the control group (Friedman test: $\chi^2 = 3.9$, $p = 0.755$). This contrasted markedly with the oxytetracycline-treated group, which displayed a statistically significant peak in degranulating eosinophils at 12 wpt (Figure 8.2.1; Friedman test: $\chi^2 = 11.9$, $p = 0.042$). Furthermore, the maximum score of 3 (> 10 degranulating eosinophils per worm section) was only ever assigned to nodules in this group, and moreover was consistently observed between 4 and 48 wpt.

8.2.2. Factors associated with eosinophil degranulation

Immunohistochemical staining for WSP in all sections between 8 and 24 wpt enabled the semi-quantitative assessment of *Wolbachia*-specific precipitate (Figure 8.2.2). As expected, *Wolbachia* was abundant in the lateral hypodermal cords and reproductive system of healthy, reproductively-active female worms removed from non-treated control animals [Figure 8.2.2 *(a)*]. Sections from the melarsomine-treated group also revealed intense staining for WSP, despite evidence of worm morbidity [Figure 8.2.2 *(b)*]. In contrast, there was a marked reduction in WSP staining, demonstrating a decrease in *Wolbachia*, in sections from the oxytetracycline-treated group [Figure 8.2.2 *(c)*], although production of microfilariae and anatomically normal internal structures were still apparent in some sections.

The study of worms from non-treated controls (Figure 8.2.3) provided a reference for the structural characteristics that could be identified and compared across all sections using a simplified version of a form proposed by Striebel (1988). Control worms

were found to exhibit a smooth turgid cuticle [Figure 8.2.3 *(a)*], and the underlying hypodermis was uniform with nuclei present [Figure 8.2.3 *(b)*]. *Wolbachia*-specific precipitate was present in the hypodermal cords and in the reproductive apparatus, and the presence of microfilariae (or other intact embryonic stages) provided further evidence of worm viability [Figure 8.2.3 *(b)*]. In contrast, worms that showed signs of morbidity exhibited a rough and broken cuticle, a vacuolated hypodermis and degenerating reproductive apparatus [Figure 8.2.4 *(a)*]. This degree of pathology was unusual for an oxytetracycline treated nodule at 8 wpt; however, although eosinophil infiltration had occurred, the cells were not adherent to the cuticle and in addition, there was no intense degranulation [Figure 8.2.4 *(b)*]. Conversely, with morphologically-normal sections from the oxytetracycline group at 12 wpt (Figure 8.2.5), a cluster of degranulating eosinophils was evident adjacent to the worm cuticle [Figure 8.2.5 *(a)*]. The placement of a digital zonal scale facilitated the spatial semi-quantification of factors related to worm viability that could influence the degranulation of eosinophils [Figure 8.2.5 *(a)*]. Deposition of eosinophilic granules was evident amongst the Splendore-Hoeppli material [Figure 8.2.5 *(b)*] and directly against the cuticle [Figure 8.2.5 *(c)*] of oxytetracycline-treated, but apparently viable, worm sections.

Eighty-nine per cent of eosinophil clusters (124 clusters) that met the inclusion criteria for statistical analysis (>10 eosinophils per cluster) were found in oxytetracycline-treated nodules. Comparison with semi-quantitative neutrophil scores in nodules removed at 8 wpt revealed a weak but significant negative correlation with degranulating eosinophils (Figure 8.2.6, Spearman's ρ: $n = 135$, $p = 0.002$). Also at 8 wpt, there were significant, positive relationships between the degranulating eosinophil score and a) the presence of worm sections in the

microscopic field (Fisher's exact test; $p = 0.020$); b) the proximity of worm sections (Spearman's ρ; $p = <0.001$); and c) scores denoting normal morphological features in the worm sections (Table 8.1). However, there were no significant associations between degranulating eosinophils and intrauterine microfilariae. There were also no statistically significant relationships at the other two time points, since almost all worm sections were abnormal and thus the statistical power of the analyses was suboptimal.

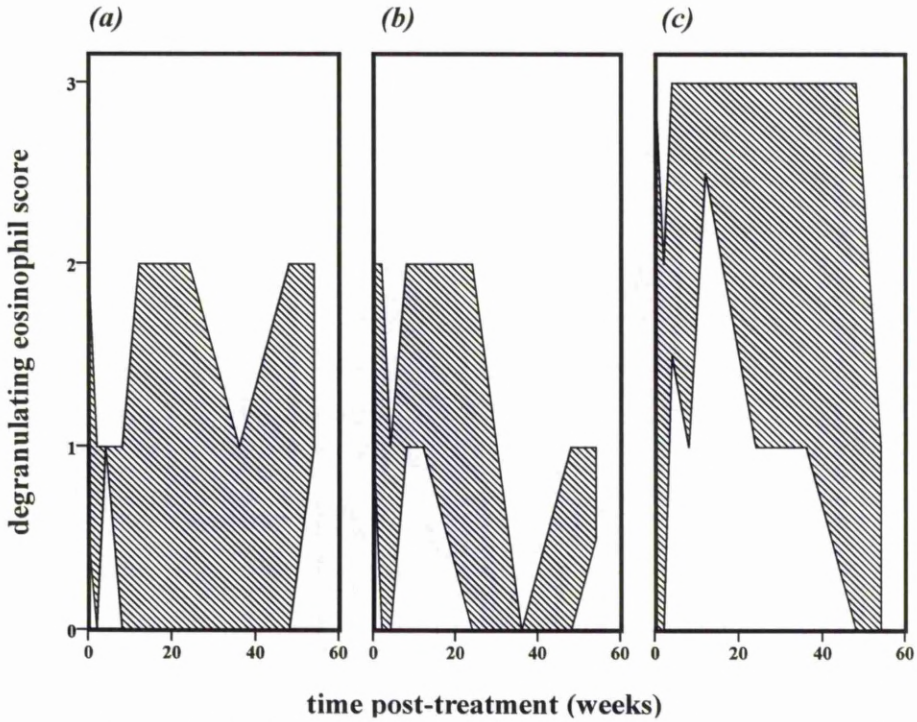


Figure 8.2.1. Semi-quantification of degranulating eosinophils in transects of *O. ochengi* nodules in *(a)* non-treated control, *(b)* melarsomine-treated or *(c)* oxytetracycline-treated animals. Degranulating eosinophils were scored per worm section on a four-point scale (0 = 0, 1 = <1, 2 = 1 - 10, 3 = >10). The shaded area represents the region bounded by the median and maximum scores.

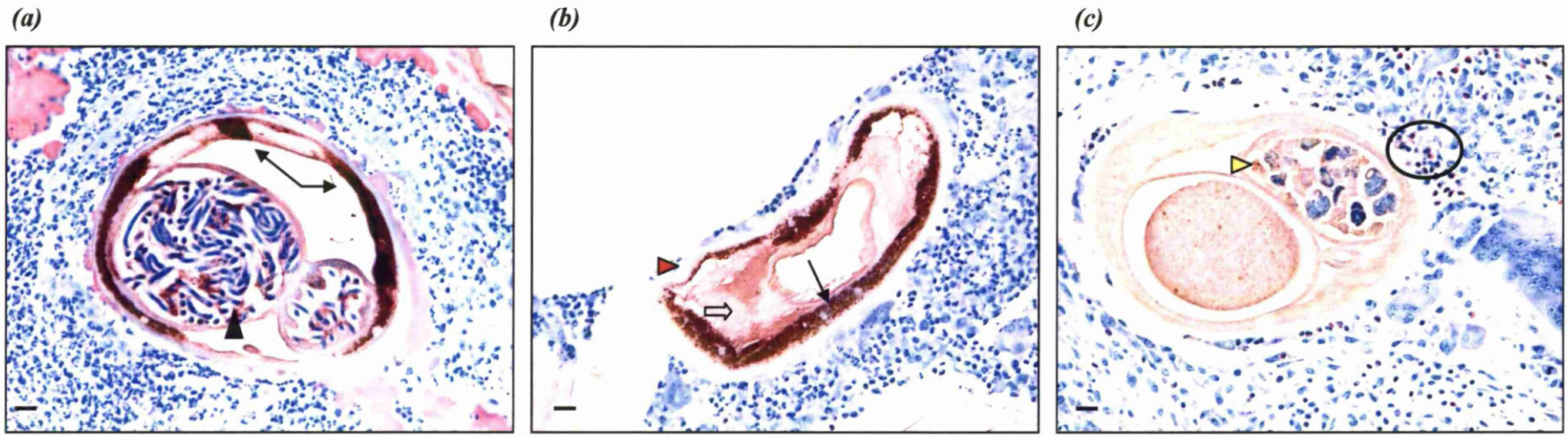


Figure 8.2.2. Identification of *Wolbachia* in *O. ochengi* onchocercomata removed from *(a)* no-treatment control, *(b)* melarsomine-treated, or *(c)* oxytetracycline treated animals at 12 weeks post-treatment. *(a)* Intensely stained hypodermal chords (arrows) and microfilariae (black arrowhead) inside viable female worm. *(b)* Heavily stained hypodermal chords (arrow) denoting presence of *Wolbachia* inside a pathologically altered worm. The convoluted cuticle (red arrowhead) shows signs of detachment from the underlying hypodermis and the pseudocoelomic cavity is also filled with amorphous material (open arrow). *(c)* Lack of dark brown precipitate denotes depletion of *Wolbachia* in the lateral hypodermal chords of this female worm. Note normal morphology indicated by the turgid cuticle attached to the nucleated hypodermis, but also signs of embryonic degeneration (yellow arrowhead). Degranulating eosinophil clusters are also evident (circled). Stain: anti-WSP antibody with 3,3-diaminobenzidine precipitate; Giemsa counterstain. Scale bars represent 20 μm.

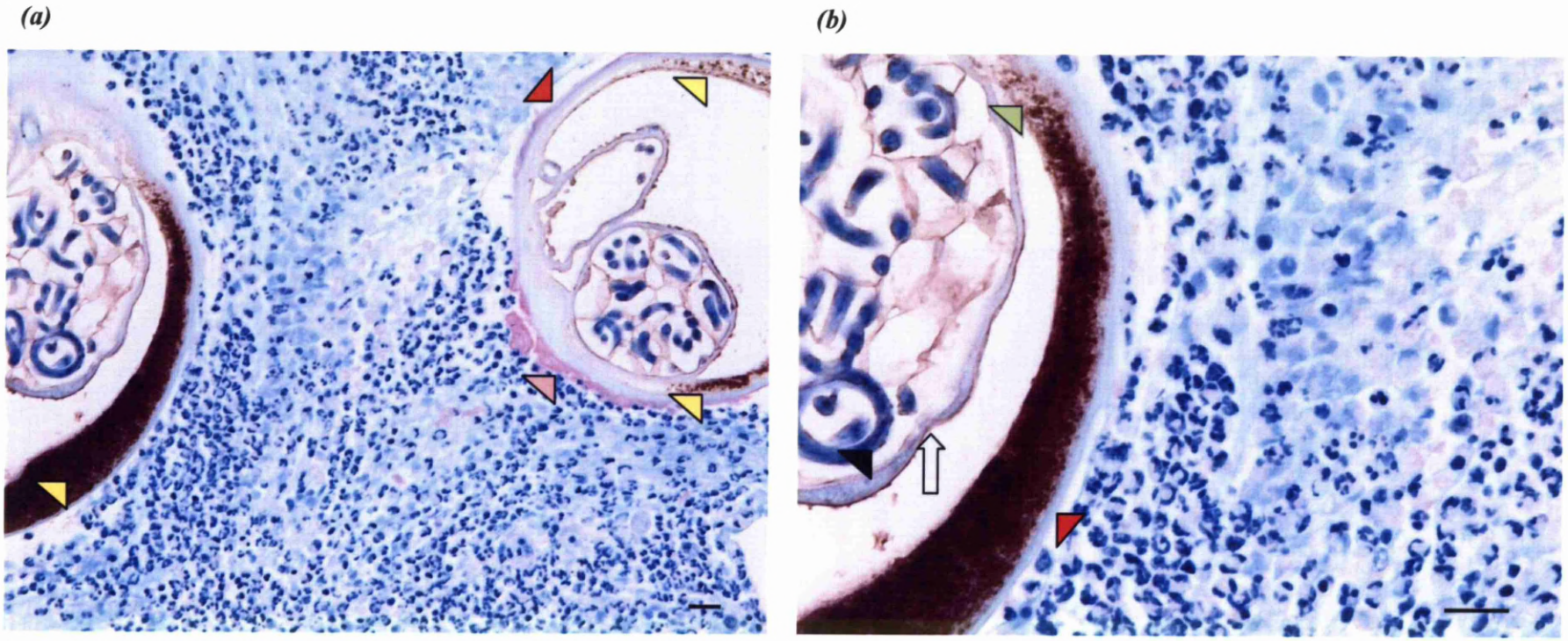


Figure 8.2.3. Structural characteristics of viable, reproductively active female *O. ochengi* from an untreated control animal. Note the smooth cuticle (red arrowheads) adherent to the underlying, non-vacuolated hypodermis (green arrowhead), *Wolbachia*, evidenced by the brown precipitate, are distributed in the lateral hypodermal chords (yellow arrowheads), and are also present in the intrauterine microfilariae (black arrowhead). The uterine wall (open arrow) is smooth. Splendore-Hoeppli deposits (pink arrowhead) are also present. Stain: anti-WSP antibody with 3,3-diaminobenzidine precipitate ; Giemsa counterstain. Scale bars represent 20 μm.

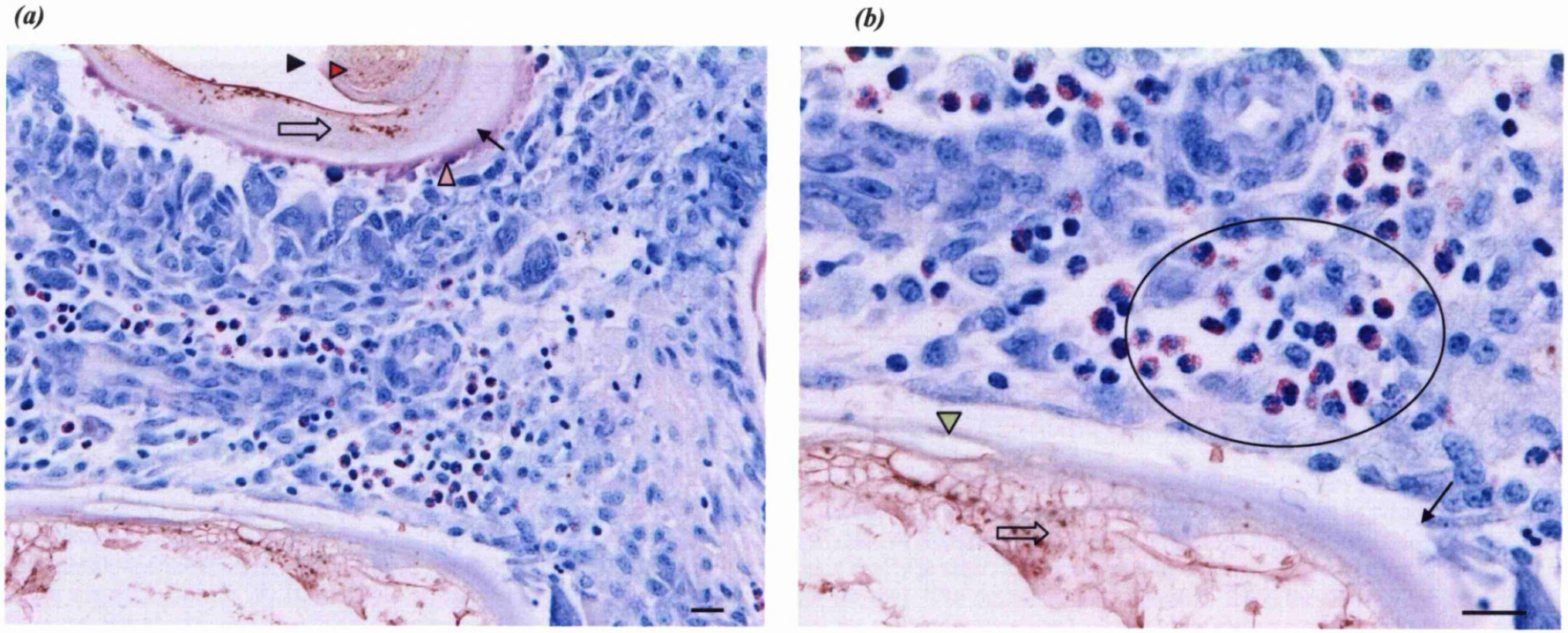


Figure 8.2.4. Section from an oxytetracycline-treated *O. ochengi* nodule removed at 8 wpt. ***(a)*** Two worm sections display different levels of pathology. The upper section is surrounded by Splendore-Hoeppli deposits (pink arrowhead), and the worm is bounded by a thin cuticle that is broken in places (arrow). The hypodermal chords contain few *Wolbachia* (open arrow). Remnants of the reproductive apparatus are present but filled with amorphous material (red arrowhead), and the uterine wall is broken (black arrowhead). The pseudocolemomic cavity is clear. The lower section is clearly degenerating, and as seen at higher magnification in ***(b)***, the cuticle is rough and indistinguishable in places (arrow). The cuticle is also detached from the underlying hypodermal structures (green arrowhead), which have become vacuolated, and few *Wolbachia* are visible (open arrow). Eosinophils are present (encircled), but are not adhered to the cuticle and there are no signs of extensive degranulation. Stain: anti-WSP antibody with 3,3-diaminobenzidine precipitate; Giemsa counterstain. Scale bars represent 20 μm.

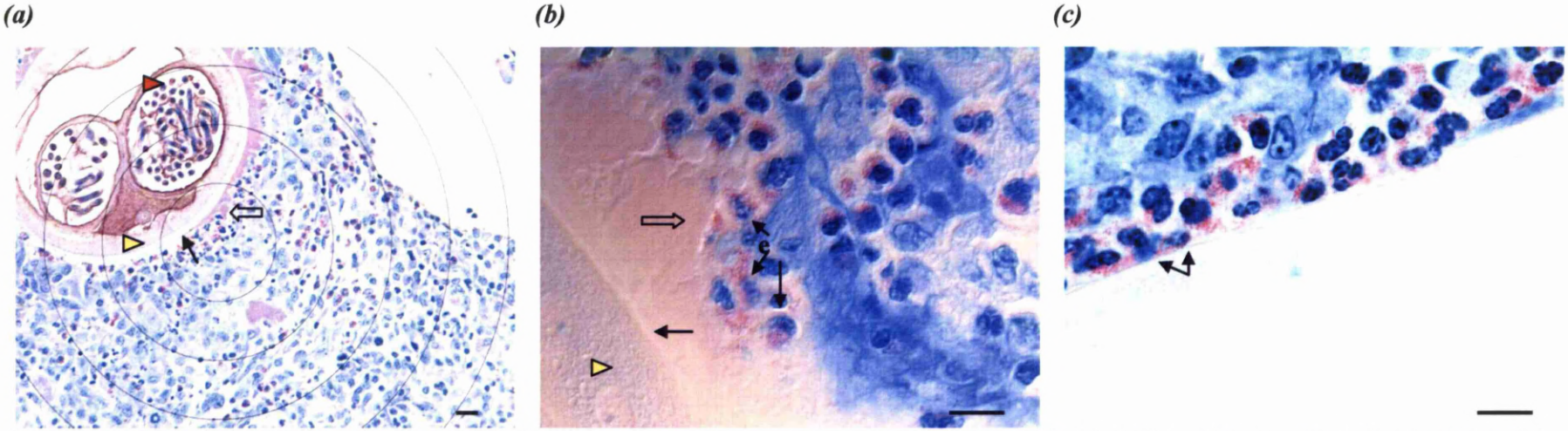


Figure 8.2.5. Assessment of degranulating eosinophil clusters and their association with indicators of filarial vitality within oxytetracycline-treated nodules (sections from 12 wpt). ***(a)*** Clusters were identified and overlaid with a digital zonal template (50 μm intervals). The main eosinophil cluster (central zone) is adjacent to the cuticle (arrow) and intermingled with Splendore-Hoeppli deposits (open arrow). The cuticle is rough but intact and adherent to the uniform underlying hypodermis (yellow arrowhead), which is depleted of *Wolbachia*. Paired uteri contain abundant microfilariae, with some signs of degeneration (red arrowhead). Stain: anti-WSP antibody with 3,3-diaminobenzidine precipitate; Giemsa counterstain. Scale bar represents 20 μm. ***(b)*** Phase contrast image of degranulating eosinophils [**e**] adjacent to Splendore-Hoeppli deposits (open arrow). The worm cuticle (arrow) is smooth and adherent to the underlying hypodermis (yellow arrowhead). Giemsa stain. Scale bar represents 10 μm. ***(c)*** Intense activity of degranulating eosinophils in contact with smooth worm cuticle (arrows). Giemsa stain. Scale bar represents 10 μm.

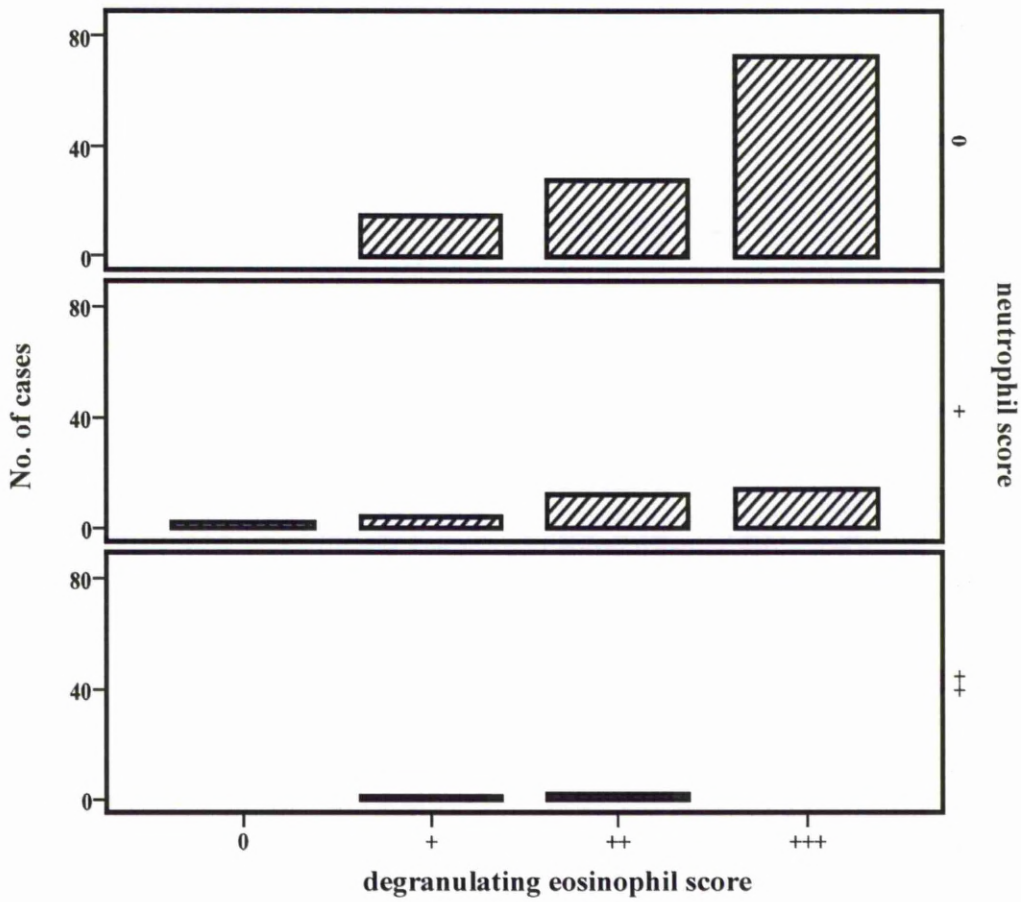


Figure 8.2.6. Correlation between degranulating eosinophil scores and neutrophil scores in sections removed at 8 wpt. Cases represent all regions containing clusters of >10 eosinophils and their peripheral zones within a 150 μm radius. Semi-quantitative-scoring system: +: scant, ++: few, +++: many cells observed.

Table 8.1. Associations between presence and integrity of *O. ochengi* adult worm sections in bovine nodules and degranulating eosinophil (DE) score at eight weeks post-treatment.

observation	scoring system	value of χ^2 or r_s	*n*	association with DE score, *p*
presence of worm	absent =0; present =1	9.319†	135	**0.020**
proximity to worm (if present)	zone 0: >50; zone 1: >50, <100; zone 2: >100, <150	-0.334b*	120	**<0.001**
genital tract integrity	damaged wall = 0; smooth wall = 1 (normal)	14.745†	43	**<0.001**
cuticle integrity	broken = 0; rough = 1; smooth = 2 (normal)	0.228*	115	**0.014**
hypodermal integrity	vacuolated = 0; thin = 1 (normal)	9.993†	111	**0.011**
reproductive status	non-productive = 0; productive = 1	7.301†	109	**0.026**
intrauterine microfilariae	none = 0; present, damaged = 1; present, normal = 2	0.089*	113	0.350

†Fisher's exact test.

*Spearman's ρ.

Bold type indicates statistical significance. The associations between degranulation and worm integrity were positive in all cases; i.e. DE scores were moderate and/or high where the sections exhibited normal structure. Sample sizes varied between analyses because only worm sections that could be scored unambiguously for each characteristic were included. Zone boundaries were measured in micrometres.

8.3. Discussion

The morphological evaluation of the health and reproductive viability of *O. ochengi* was based on the proposed evaluations tabulated by Striebel (1988), and simplified to enable accurate assessment while achieving a high throughput of sections. The evaluation of the cuticle and hypodermis was particularly significant, because as these structures were present throughout the length of all worms, their morphological status could be assessed in all sections. Early pathological signs are manifested by vacuolation of the hypodermis, but this can also be a sign of ageing (Copeman, 1979). Fragmentation of the cuticle is reported to occur at a later stage of decomposition (Copeman, 1979); thus when taken together, these observations are a reliable indicator of worm morbidity (Striebel, 1988).

It is acknowledged that bias was a potential compromising factor with this set of data because firstly, only sections between the time-points of 8 and 24 weeks were processed. This was because the previous, detailed full quantification of granulocytes from nodule transects removed at each time point revealed that the major changes in cell composition were happening between these time-points. Secondly, we targeted eosinophil clusters within each section, prior to the assessment of localized features surrounding each cluster. A statistically more robust approach would have encompassed the random assessment of sections, and recorded the cellular composition and worm vitality within. However, because large eosinophil clusters were almost exclusively found in the oxytetracycline group, the examination of hundreds of microscopic fields would have been required to obtain a sample size sufficient to elucidate the factors influencing eosinophil degranulation, and time restrictions prevented this approach. Nevertheless, each section was screened 'blind' for the presence of eosinophil clusters, and the criteria for the assessment of worm

vitality were standardized as far as possible. Furthermore, because the evaluation of worm vitality was assessed using a variety of features, a semi-quantitative approach was deemed reliable (Striebel, 1988), and thus non-parametric statistical tests were applied. Despite this, it is important to emphasise that our methodology could not eliminate the possibility that inter-animal variability (*e.g.,* in immune reactivity) was responsible for the apparent relationships between eosinophil degranulation and worm vitality. For instance, if animals with potent degranulation responses had had greater numbers of reproductively-active worms within nodules before treatment began, this could have compromised the analyses.

The time-points examined represent "snapshots" of an *in-vivo* process which is finely orchestrated in the immune response to *Wolbachia*-depleted worms. The results indicate that the 8 wpt time point was the most critical for eosinophil activation, as it preceded the degranulation peak (Figure 8.2.1). In the oxytetracycline treated group, morphologically-normal (and reproductively viable) worms were commonly observed at 8 wpt, in association with eosinophil clusters. This seems consistent with an increase in eosinophil chemotaxis and activation occurring towards normal, but *Wolbachia*-depleted worms, because eosinophils were not activated in the presence of already-damaged worms. At 12 wpt, eosinophil degranulation was at its peak in the oxytetracycline-treated group. Most worms observed showed signs of deterioration, indicating that eosinophil cytotoxic products were damaging the worm surface; but some worms were still morphologically normal (smooth cuticle and thin, unvacuolated hypodermis) and were attracting degranulating eosinophils. Eosinophils observed in melarsomine-treated sections were not degranulating extensively, and oxytetracycline-treated worms that were depleted of *Wolbachia* and showed signs of internal degeneration did attract

eosinophils, but they remained distal to the cuticle surface and did not degranulate as intensely. The lack of any significant associations between degranulating eosinophils and the measures of worm viability at 12 and 24 wpt was not surprising, as there were very few worms remaining with normal physiological features and thus the expected frequencies in the cross-tabulation analyses were extremely low.

Interestingly, eosinophilic adherence to structurally intact microfilariae has been reported before in onchocerciasis patients with localized hyper-responsiveness [sowda (Buttner and Racz, 1983)]. Moreover, in the same report it was noticed that in the region of degenerating microfilariae, there was eosinophilic material, but the main cellular type was the macrophage (Buttner and Racz, 1983). In patients with sodwa, neutrophils have been shown to be hyporesponsive (Rubio de Kromer et al., 1995) , and since nodules reduce in size and become infiltrated with eosinophils, this may represent the closest natural analogue of the immune response that is seen in oxytetracycline-treated individuals.

Eosinophil-parasite adherence was also reported after treatment with DEC, with eosinophils observed adhering tightly to microfilarial cuticles, accompanied by the deposition of granules onto the parasite surface (Racz et al., 1982). Their adhesion on to the surface of *O. volvulus* L3 may be mediated by complement (Wildenburg et al., 1996) or via parasite-specific receptors such as fibronectin receptors, which adhere to fibronectin on the larval epicuticle (Brattig et al., 1995). Eosinophils were also reported surrounding microfilariae in abundance within hours of topical application of amocarzine, but whether the initial attraction was to live or degenerated microfilariae could not be determined, as amocarzine was reported to exert damage to microfilariae within this timeframe (Gutierrez-Pena et al., 1998).

In-vitro eosinophil adherence to live ascarid larvae has been reported in guinea pigs (Rockey et al., 1983), and for *Brugia pahangi*, complement-mediated eosinophil adherence and degranulation occurred on viable microfilariae *ex-vivo* when incubated with cat (Johnson et al., 1981) and rat (Chandrashekar et al., 1985) granulocyte populations.

Studies examining the effect of eosinophils in *Echinococcus granulosus* patent infections suggested that eosinophils degranulate at the host-parasite interface, because the eosinophil granule products ECP and EDN were found in the hydatid cyst wall, and were also present inside the hydatid cysts (Ramos et al., 2006). Furthermore, the expression of compound exocytosis by eosinophils is thought to aid in the directed release of granule products towards an infected site, thus protecting host tissues from cytotoxic granule contents (Hafez et al., 2003). Directed degranulation could be the basis of the difference between the early eosinophil activation against viable worms as evidenced here, and the later involvement of eosinophils in the clearance of worm fragments, as reported in Chapter 6.

9. Mechanisms of filarial killing by eosinophils in cattle treated with oxytetracycline.

9.1. Introduction

The contribution of eosinophils towards filarial death was studied using electron microscopy, to evaluate eosinophil activation in relation to filarial cuticular damage. Quantitative assessment of nodule area was used as a gross indicator of worm viability, in addition to *Wolbachia* 16S rRNA:*Oo*GST1a transcript ratios to assess the impact of macrofilaricidal treatment on *Wolbachia* depletion and filarial viability.

The *in-vitro* killing of filarial larval stages by eosinophils has been previously reported. Strote et al (1990) captured electron micrographs of eosinophils depositing their granules onto *O. volvulus* microfilariae and Greene et al (1981) demonstrated the activation and attachment of eosinophils to larvae. As these data were obtained *in-vitro*, however, and although they showed that larval killing was enhanced in the presence of immune serum, the role of eosinophils in filarial killing *in-vivo* could only be inferred. Histological evidence of larval killing *in-vivo* reported in the literature include the deposition of eosinophil products onto dermal microfilariae (Ackerman et al., 1990), and increased lymphatic infiltration of eosinophils concomitant with microfilarial degeneration (Racz et al., 1982; Wildenburg et al., 1994) following filaricidal treatment. In a surrogate host, indirect evidence of eosinophil-mediated killing of microfilariae was also demonstrated via the delay in *O. lienalis* clearance in mice after the blocking of IL-5 (Folkard et al., 1996).

In studies assessing the efficacy of vaccination against filarial infection, several reports have identified that eosinophils are critical in the immune response following immunisation with irradiated L3. In the chamber model of *O. volvulus*, there was a significant increase of activated eosinophils recovered from chambers within 3 days of larval challenge (Lange et al., 1994). Eosinophil infiltration was dependent on the presence of IL-4 and IL-5, and larval killing was only achieved when eosinophils came into contact with the larval surface (Lange et al., 1994). In another study in the same model, mice that were treated with antibodies to eliminate eosinophils were not able to mount an immune response post challenge, which led to increased parasite survival (Abraham et al., 2004). The elimination of IgE from this model also had a negative impact of eosinophil recruitment (Abraham et al., 2004). In *L. sigmodontis*, eosinophil-mediated larval killing post challenge was also identified in mice vaccinated with irradiated larvae, and a role for IL-5 was reported (Le Goff et al., 2000a). A further study using irradiated L3 of *A. viteae* for vaccination in jirds identified a direct association between eosinophil activation, degranulation and cuticular disruption within 4 days of larval challenge (Bleiss et al., 2002).

Thus, while the role of eosinophils in the death of larval-stage filariae has been well documented, their effect on adult parasites is more controversial. In histological studies of *O. ochengi*, eosinophils were observed in oxytetracycline-treated onchocercomata, but their role in adult worm killing could only be inferred (Nfon et al., 2006). In the current study, relatively frequent nodulectomies of melarsomine or oxytetracycline-treated nodules facilitated the observation of the progression of eosinophil activation and filarial damage via TEM. The TEM processing of neutrophils and eosinophils from respective enriched cell pools from bovine blood

(Section 3.3.3.1) aided the accurate identification of cells in the the nodule images (Appendix 12.6).

9.2. Results

9.2.1. Effects of treatment on nodule size and bacterial and filarial transcripts.

Gross observation of nodules during the longitudinal experiment indicated that treated nodules, in particular those in the melarsomine group, reduced in size after treatment. Measurement of section areas confirmed the observed trends, as the median nodule area gradually reduced during both the melarsomine and oxytetracycline treatments compared to untreated controls (Figure 9.2.1). The overall difference was statistically significant for the melarsomine-treated group [Figure 9.2.1 *(b)*; Friedman test: $p = 0.012$], but not for the oxytetracycline-treated group [Figure 9.2.1 *(c)*; Friedman test: $p = 0.096$], although a similar trend in the size reduction was apparent.

The quantification of *Wolbachia* (16S rRNA) and filarial (*Oo*GST1a mRNA) transcripts served to elucidate the effects of treatment on bacterial and worm host viability (Figure 9.2.2). Absolute quantification revealed a non-significant reduction of the expression of *Oo*GST1a in both oxytetracycline and melarsomine-treated nodules, which approached the critical probability only in the melarsomine group (GLM repeated measures, Tamhane's T2 post-hoc test, MEL v CON: mean difference = -2.3 log, $p = 0.069$). In contrast, 16S rRNA decreased significantly in both treatment groups when compared to the control group, and this was more extensive in the oxytetracycline group (GLM repeated measures, Tukey's post-hoc test, MEL v. CON: mean difference = 2.1 log, $p = 0.016$; OXY v. CON: mean difference = -3.1 log, $p = 0.001$).

Extirpated nodules varied greatly in size and worm content throughout the experiment, therefore the ratio between the expression of filarial *Oo*GST1a mRNA and *Wolbachia* 16Ss rRNA was calculated to provide a more accurate assessment of the nodule content compared to the unadjusted transcript counts (Figure 9.2.2). The 16S rRNA:*Oo*GST1a ratio was significantly reduced compared to the control group in oxytetracycline treated nodules only (Tamhane's T2 test: mean difference = -2.1 log, p <0.001). Furthermore, this ratio was significantly lower in the oxytetracycline-treated group than in the melarsomine-treated group (Tamhane's T2 test: mean difference = -2.4 log, p <0.001).

9.2.2. **Effect of treatment on worm ultrastructure**

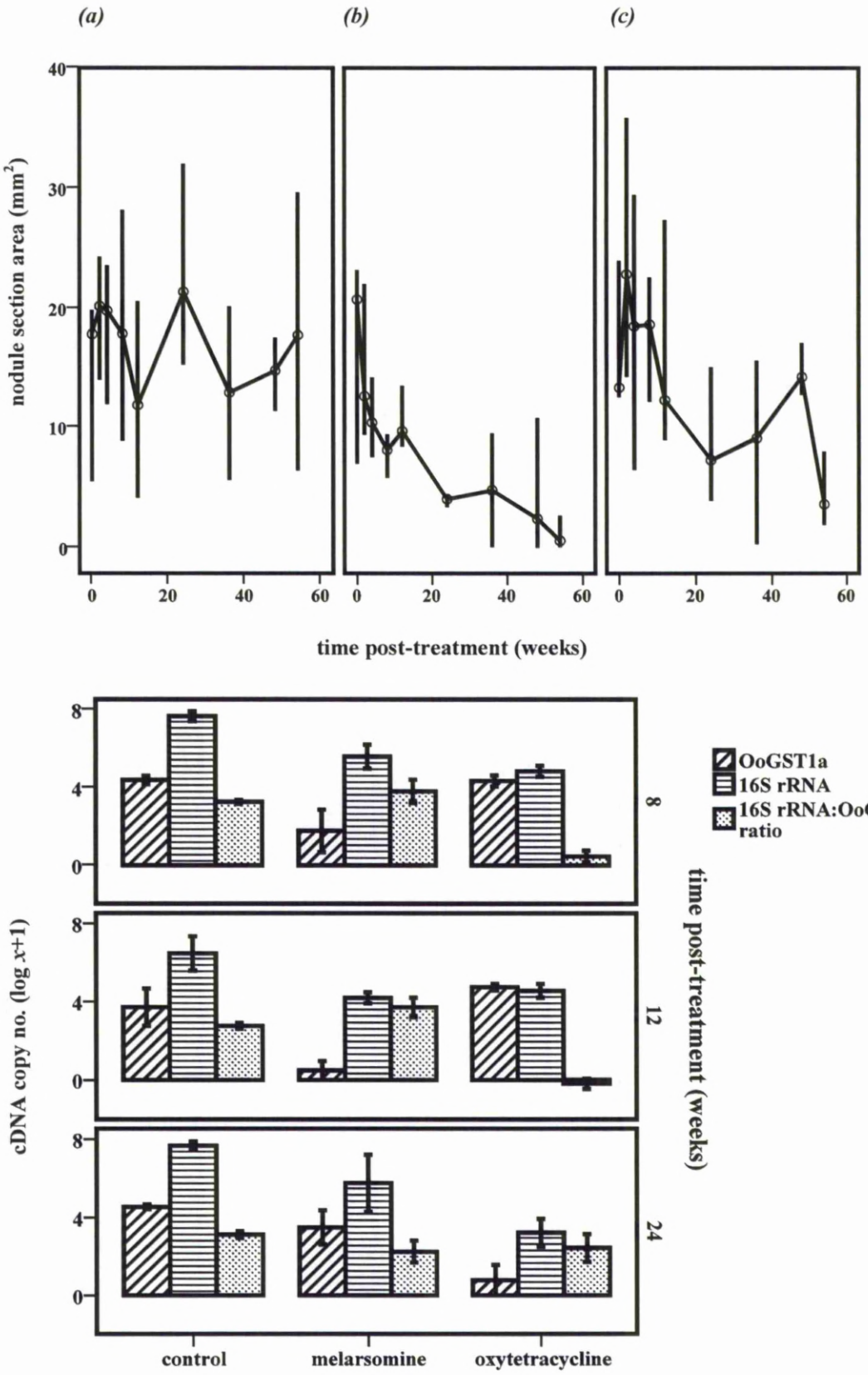
At 12 wpt, filarial sections from the untreated control group invariably contained *Wolbachia* in their hypodermal cords [Figure 9.2.3 *(a)*]. In addition, worms were often found surrounded by a predominantly neutrophilic infiltrate [Figure 9.2.3*(b)*]. The filarial ultrastructure remained normal, despite the close proximity of neutrophils to the cuticle [Figure 9.2.3 *(c)*], and the presence of exocytosed granules, which were observed frequently [Figure 9.2.3 *(d)*]. Filarial sections from the melarsomine-treated group also contained *Wolbachia* in their hypodermal cords at 12 wpt [Figure 9.2.4 *(a)*]. A mixed cellular infiltrate was often observed surrounding the worms [Figure 9.2.4 *(b)*], which included eosinophils [Figure 9.2.4 *(c)*]. These granulocytes were observed flattened against the outline of the Splendore-Hoeppli deposits [Figure 9.2.4 *(c)*], and engulfing fragments of the material with lemellopodiae [Figure 9.2.4 *(d)*]. However, degranulation was infrequent in this group, as the cells were intact and there were few exocytosed granules [Figure 9.2.4 *(d)*].

At the same time point (12 wpt), the contents of the oxytetracycline-treated nodules appeared markedly different to those in the other experimental groups. The lateral hypodermal cords featured evidence of *Wolbachia* degradation and depletion [Figure 9.2.5 *(a)*]. Eosinophils were commonly observed adjacent to the cuticle, and they were also found inside the pseudocoelomic cavity [Figure 9.2.5 *(b)*]. These eosinophils were clearly activated, as pseudopodia were observed extending into cuticular invaginations [Figure 9.2.5 *(c)*], where cuticular deformation and thinning was evident [Figure 9.2.5 *(d)*]. Activation of eosinophils was also apparent by the polarisation and adherence of cells to the cuticular surface, and the foamy appearance of the granules which were in various stages of detachment from vesicular membranes [Figure 9.2.5 *(e)*]. On closer inspection, granule membranes were observed adhering to the cuticular surface coat [Figure 9.2.5 *(f)*].

Following page:

Figure 9.2.1. Section areas of nodules removed during the longitudinal study from *(a)* non-treated control, *(b)* melarsomine-treated and *(c)* oxytetracycline-treated animals. Lines represent the median; bars represent the range.

Figure 9.2.2. Quantitative gene expression of Onchocerca ochengi glutathione S-transferase 1a (OoGST1a) mRNA, *Wolbachia* 16S rRNA, and the 16S rRNA:OoGST1a ratio in onchocercomata from untreated controls, and oxytetracycline or melarsomine treatment groups at 8, 12 and 24 wpt. Bars represent the mean ± SE.


(a)
(b)
(c)
nodule section area (mm²)
40
30
20
10
0
0
20
40
60
time post-treatment (weeks)
cDNA copy no. (log x+1)
8
4
0
8
12
24
time post-treatment (weeks)
OoGST1a
16S rRNA
16S rRNA:OoGST1a ratio
control
melarsomine
oxytetracycline

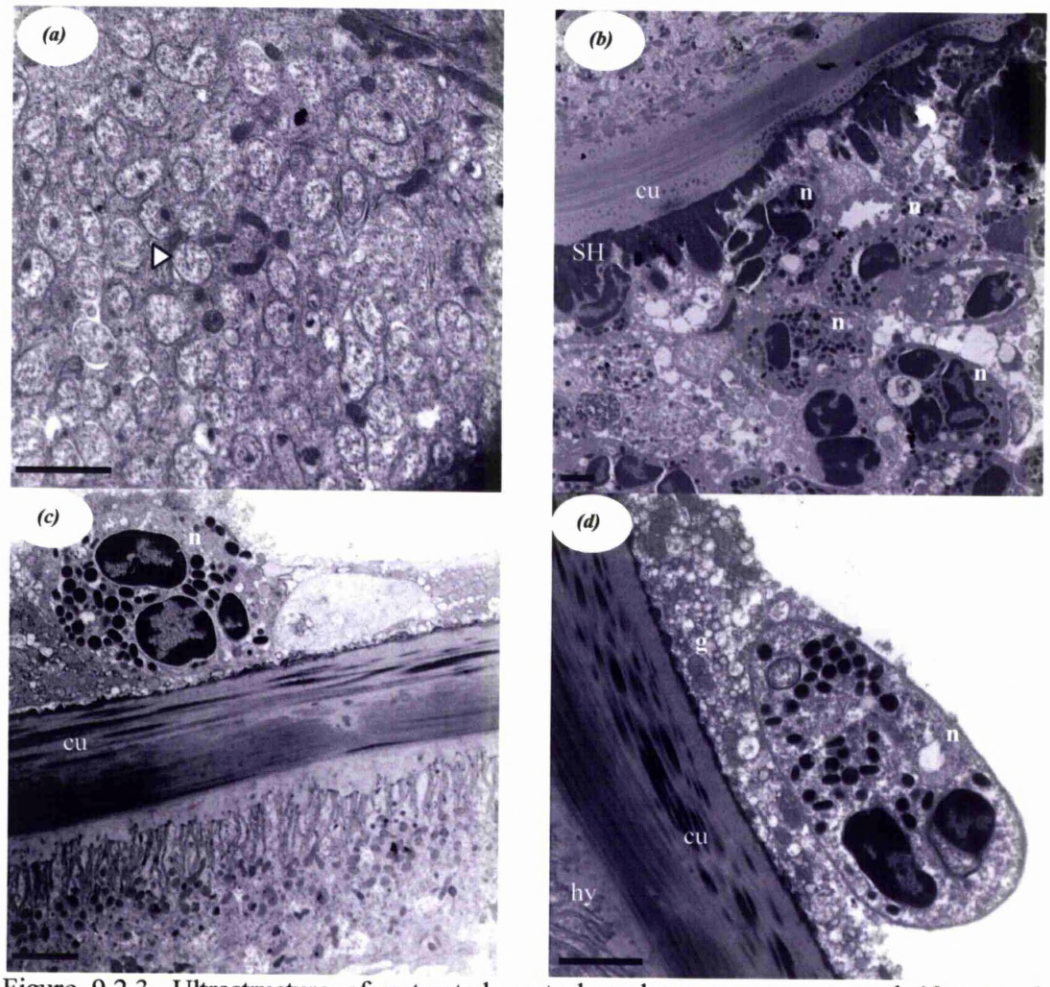


Figure 9.2.3. Ultrastructure of untreated control onchocercomata removed 12 wpt. ***(a)*** Abundant *Wolbachia* (white arrowhead) present in lateral hypodermal cords. ***(b)*** Predominantly neutrophilic (n) infiltrate adjacent to worm cuticle (cu) and Splendore-Hoeppli deposits (SH). ***(c)*** Intact neutrophil (n) in close proximity to worm cuticle (cu). ***(d)*** Elongated neutrophil (n) partly embedded in free granules (g); in close contact with morphologically normal cuticle (cu) and underlying hypodermis (hy). Scale bars represent 2 μm.

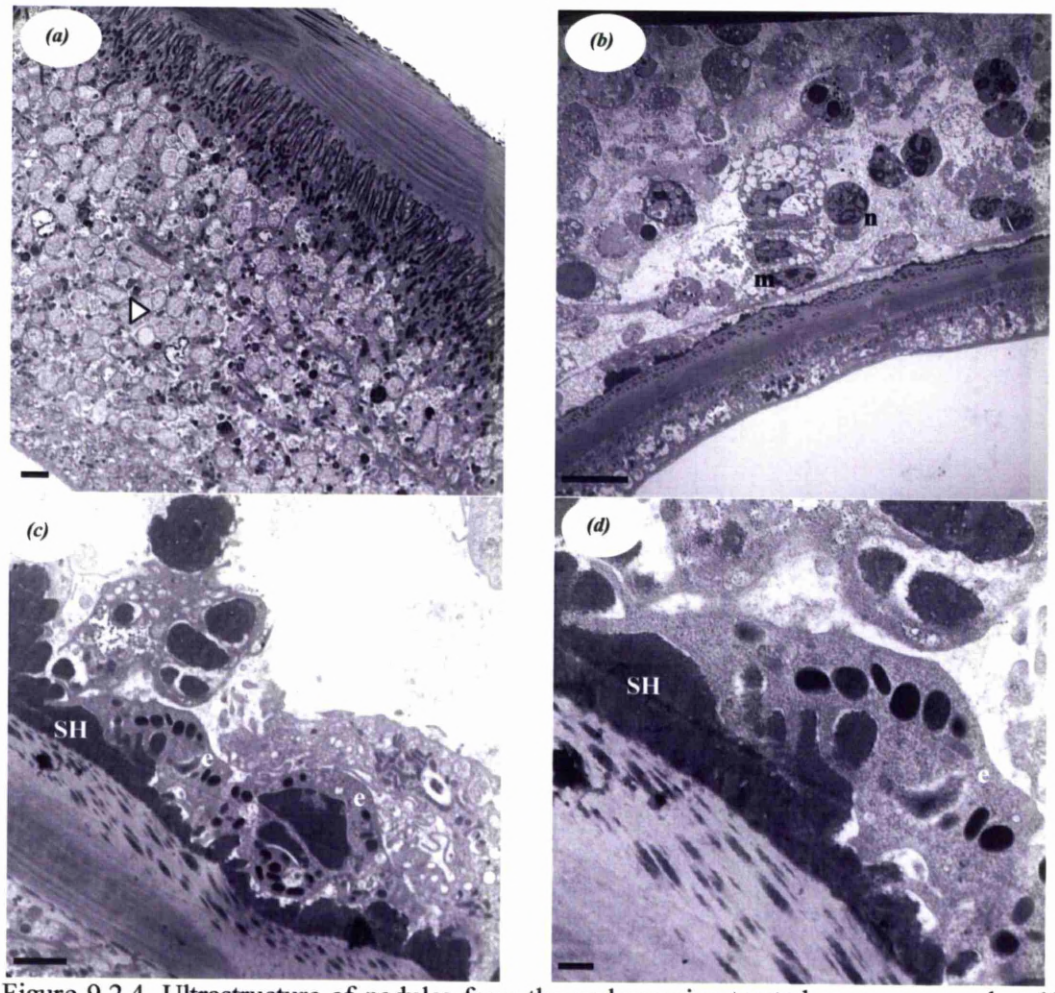


Figure 9.2.4. Ultrastructure of nodules from the melarsomine-treated group removed at 12 wpt. (a) Abundant Wolbachia (white arrowhead) in the hypodermal cords. (b) Mixed cellular infiltrate surrounding worm cuticle [macrophage (m); neutrophil (n)]. (c) Eosinophils (e) adjacent to Splendore-Hoeppli deposits (SH). (d) Eosinophil (e) flattened against Splendore-Hoeppli material (SH). Scale bars (a,c) 2 μm; (b) 10 μm; (d) 500 nm.

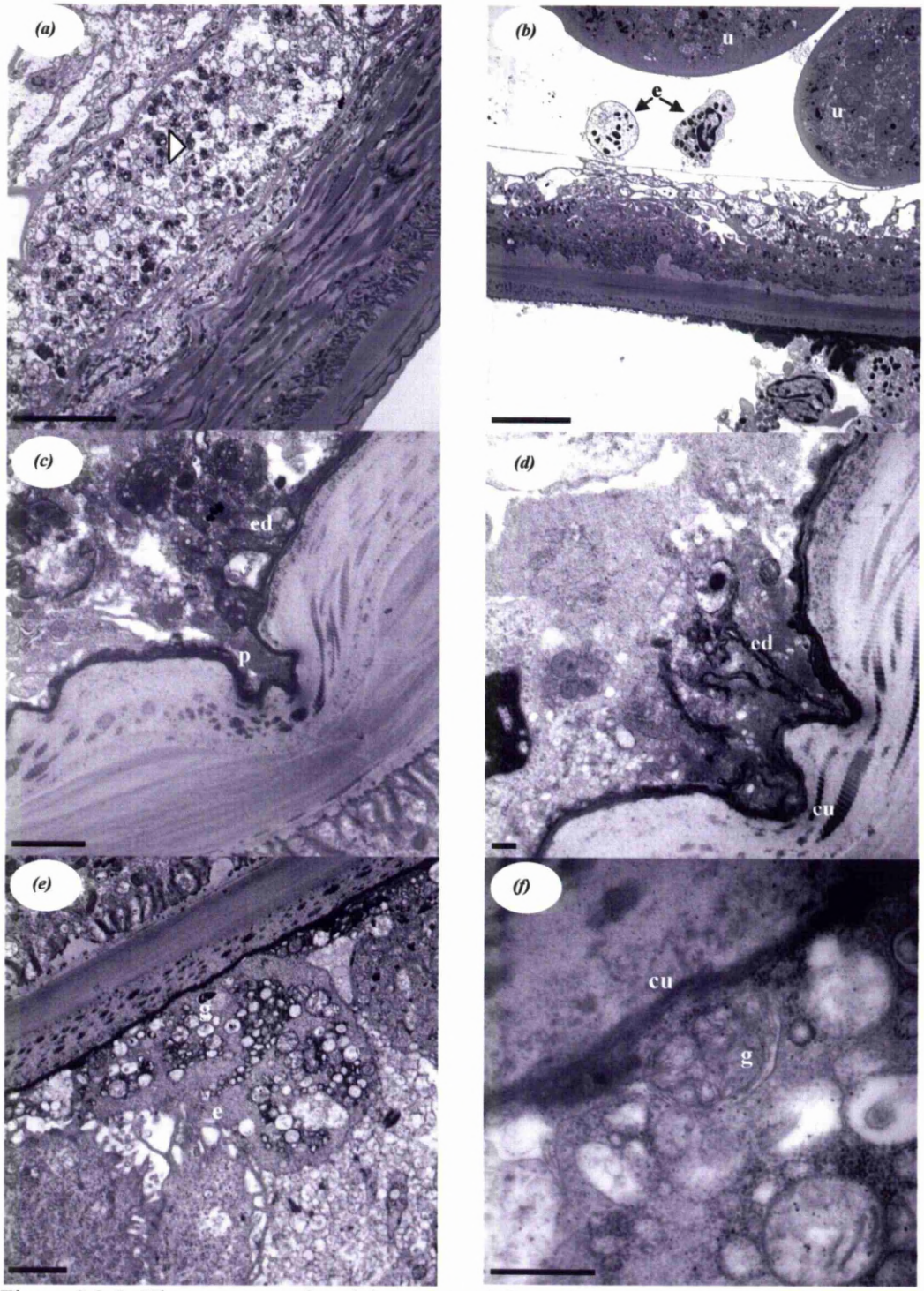


Figure 9.2.5. Ultrastructure of nodules removed from the oxytetracycline-treated group 12 wpt. *(a) Wolbachia* remnants in the hypodermal cords (white arrowhead). *(b)* Eosinophils (e) in the pseudocoelomic cavity of a female worm [note paired uteri (u)]. *(c)* The pseudopodium (p) of a degranulating eosinophil entering a cleft in the cuticle. Electron-dense (ed) granular material is indicative of granule deposits and the electron-lucent vacuole (v) reveals that degranulation has occurred *(d)* Electron–dense (ed) granule deposition on to the thinning cuticle (cu). *(e)* Vacuolated necrobiotic eosinophil (e) with hypodense granules (g), stretched along the cuticular surface. *(f)* Deposition of eosinophil granule (g) contents onto the cuticle (cu). Scale bars: (a,b) 10 μm; (c,e) 2 μm; (d,f) 500 nm.

9.3. Discussion

A reduction in onchocercomata size in synchrony with worm degeneration and resorption, in addition to a decrease in *Oo*GST1a mRNA transcripts in both treatment groups, indicated that worm death occurred after treatment with melarsomine or oxytetracycline. The rate of killing of the two filaricidal drugs was comparable to previous studies in the *O. ochengi* model. Tchakouté et al. (2006) reported that melarsomine caused total elimination of adult worms within 5 months post-treatment, evidenced by an extensive reduction in palpable nodules, plus nodule resolution and a complete absence of worm viability in all residual nodules examined microscopically. In addition, our results from the oxytetracycline-treated group were similar to earlier studies that showed a decrease in worm viability (using the MTT formazan reduction assay) from 3 months post-treatment, and mass resolution of nodules by 9 months post-treatment (Langworthy et al., 2000). Gilbert et al. (2005) reported a gradual decrease in nodule diameter after prolonged (COM) treatment with oxytetracycline, consistent with our results. Worm viability also declined gradually (assessed by MTT formazan reduction), with a considerable reduction from 12 wpt (assessed by motility scoring) in the COM treatment group (Gilbert et al., 2005).

Our data showed that in the melarsomine group, worm structure was clearly compromised in histological sections from 8 - 24 wpt; thus, it was surprising that *Oo*GST1a did not decrease more distinctly at 24 wpt. Possibly, dying worms may upregulate *Oo*GST1a in an attempt to detoxify melarsomine before they are finally killed, since glutathione *S*-transferases have a well-established role in the metabolism of xenobiotics (Pemberton and Barrett, 1989). Nevertheless, when compared as a ratio with *Wolbachia* 16S rRNA, it was evident that worm death was

not preceded by *Wolbachia* depletion in melarsomine-treated nodules. In contrast, in the oxytetracycline group, *Oo*GST1a levels dropped markedly between 12 and 24 wpt in accordance with a gradual loss of *Wolbachia*. This suggests that *Wolbachia* numbers may have fallen below a critical threshold required for worm survival; therefore, worm death due to endosymbiotic loss was apparent. However, the additional ultrastructural evaluation of nodules from the same time-points indicated that the mammalian host immune system actively participated in worm destruction in the oxytetracycline-treated group.

Electron microscopy was a powerful tool for the elucidation of eosinophil involvement in worm destruction. In the oxytetracycline-treated worms, eosinophils appeared to be activated and were observed adhering to the worm cuticle and releasing granule contents. Although eosinophils were also observed in melarsomine-treated nodules, they did not show signs of active degranulation and the integrity of the worm cuticle was maintained in their presence. This strongly supports the hypothesis that eosinophils contribute to worm death *in-vivo* after the depletion of *Wolbachia* by oxytetracycline chemotherapy.

In summation, our data indicated that in the oxytetracycline group, gradual *Wolbachia* depletion occurred. Once a critical threshold was reached, the worms were left vulnerable to attack by host eosinophils, which caused active destruction of their cuticles by cytotoxic granules. Thus, between 8 and 24 wpt, a range of worm states were observed. Worms at 8 wpt were typically morphologically normal, but were subjected to intense attack by activated eosinophils. In the subsequent time points, active killing became less common in parallel with a decrease in filarial vitality, and extensive cuticular damage had occurred. This led to the hypothesis that

active killing was driven by signals from live intact worms that were depleted of *Wolbachia*. As worm vitality decreased (due to the degranulation of cytotoxic products), the worm signal diminished and eosinophil activation subsided, probably to curtail damage to host tissue from cytotoxic granule products.

Eosinophil granule contents are capable of causing severe damage to helminth structure. Major basic protein (Kephart et al., 1984), together with MPO from neutrophils (Gutierrez-Pena et al., 1996), have both been implicated in the killing of *O. volvulus* microfilariae following DEC chemotherapy, and ECP was discharged around microfilariae after amocarzine treatment (Gutierrez-Pena et al., 1998). In addition, MBP, EDN and ECP all contributed to the death of the larval stages of *B malayi* and *B. pahangi in-vitro* (Hamann et al., 1990). In *Echinococcus granulosus* cysts, ECP released by eosinophils *in-vivo* at the host-parasite interface reached concentrations that were considered sufficiently high to damage the developing parasites (Ramos et al., 2006).

Eosinophil products EPO and MBP were found to be fundamental in the immune response against *L. sigmodontis* in mice, as knockout mice for either of these granule proteins experienced higher worm burdens (and greater worm development) compared to wild-type mice (Specht et al., 2006). In addition, EPO and MBP were found to influence the cytokine milieu in infected mice. In EPO knockout mice, an increase in IL-5 and thoracic eosinophilia was evident after infection, and an increase in IL-10 production from macrophages was also observed. IL-4 production was also shown to be dependent on the presence of EPO and MBP (Specht et al., 2006). Taken together, these results suggest that eosinophil granule proteins are an

important source of cytotoxicity in larval clearance, but they may also modulate other immune mechanisms by influencing cytokine expression.

Published evidence suggests that eosinophil effectiveness is enhanced, if not necessitated, by other immune factors such as complement or immunoglobulin. For example, murine eosinophils have been observed killing newborn *Trichinella spiralis* larvae *in-vitro* in the presence of immune serum (Kazura and Aikawa, 1980). The *in-vivo* killing of *Haemonchus contortus* larvae within the abomasal tissue of their natural ovine host was also attributed to activated eosinophils (Balic et al., 2006); and increased killing in the presence of complement, antibody and IL-5 *in-vitro* indicated that eosinophils exerted a more potent effect when primed (Rainbird et al., 1998).

There are relatively few reports of the anthelmintic effects of bovine eosinophils, especially in filariases. However, eosinophil-mediated damage of juvenile *F. hepatica* was demonstrated *in-vitro* in areas close to the parasite tegument that were not protected by an antigen-antibody flocculent coat (Glauert et al., 1985). Eosinophil degranulation was evidenced by the observation of electrolucent vacuoles appearing in the cytoplasm, and lamellipodia extended into tegumental pits on the parasites' surface (Glauert et al., 1985), which is similar to our findings with oxytetracycline-treated *O. ochengi*. The specific granule protein implicated in the killing of juvenile *F. hepatica* was bovine MBP, not EPO (Duffus et al., 1980), which is surprising since EPO is more abundant in bovine eosinophils (Roth and Kaeberle, 1981). Bovine eosinophils are equipped with complement receptors (Grewal and Babiuk, 1979), but their status with regards to Fc receptors for IgE is unknown. If they do display appropriate antibody receptors, however, they may be

capable of eliciting filarial damage in concert with complement and antibody, as has been reported for *O. volvulus* (Greene et al., 1981; Johnson et al., 1991).

Evidence for a host immune-mediated contribution to filarial death after anthelminthic treatment has also been reported previously *in-vivo* and *in-vitro*. Eosinophil-mediated microfilarial killing was reported in the lymph nodes of *O. volvulus*-infected individuals after DEC-treatment (Racz et al., 1982). Massive influxes of eosinophils were also observed in the lymph nodes of ivermectin-treated *O. volvulus* patients, concomitant with degenerating microfilariae which were surrounded by eosinophils that had released ECP and EPO (Wildenburg et al., 1994).

Eosinophils have been shown to be important effector cells in the establishment of protection in *L. sigmodontis*-infected mice. In a study by Martin et al. (2000), eosinophils were seen degranulating on live larvae within 10 days post infection of both wild-type and IL-5 transgenic mice (Martin et al., 2000). However the transgenic mice experienced a greater eosinophilic response, with 41 % of larvae damaged within one month of infection. Within two months post-infection, granulomas in these mice contained many eosinophils (as well as macrophages), which were found adherent to larvae. In addition, the recovery rate of adult worms was considerably reduced (Martin et al., 2000).

In addition to the well-illustrated role of eosinophils in the clearance of filarial larvae, there is also published evidence to support a role for neutrophils in the control of adult worms. In the *L. sigmodontis* mouse model, the encapsulation of adult worms within nodules was mediated by neutrophils (Al-Qaoud et al., 2000; Saeftel et al., 2001). These studies identified a role for IL-5 in the indirect activation of neutrophils via neutrophilic cytokines such as TNF-α (Al-Qaoud et al., 2000), and

a role for IFN-γ in the encapsulation and subsequent control of adult worms by neutrophils (Saeftel et al., 2001). Furthermore, in these inflammatory nodules, neutrophils were the effector cells responsible for filarial death (Al-Qaoud et al., 2000).

The apparent discrepancy regarding the involvement of neutrophils in the death of adult filariae within different systems may be due to a variety of factors. Within each system, adult worms show preference for different predilection sites within their hosts. For example, adult *L. sigmodontis* reside within the pleural cavity of murine hosts, whereas *O. ochengi* adults reside within intradermal nodules. In addition, there could be a relative difference in the functions of granulocytes in each system, since cell infrastructure and cytokine pathways are not always analogous between species. A further consideration is the relative chronicity of each parasite species. The long lifespan of *O. volvulus* and *O. ochengi* compared to *Litomosoides* may facilitate different mechanisms of immune tolerance, versus control when tolerance mechanisms are compromised. Lastly, mice are not the natural host of *L. sigmodontis*. It cannot be ruled out that any results from unnatural host-parasite systems may be aberrant and misleading (Meeusen and Balic, 2000).

The findings within this study are remarkable because our data indicate that in the natural host of *O. ochengi*, eosinophils can be actively involved in the destruction of adult worms, which is in itself controversial (Meeusen and Balic, 2000). Adult female *Onchocerca ochengi* and *O. volvulus* filariae do not migrate, so they are not exposed to a continuously changing host tissue environment. Furthermore, larval filarial stages undergo moulting, and it has already been shown that eosinophils do not attack the L4 stage (Brattig et al., 1991), which suggests that different parasite

stages express different antigens. Thus, perhaps the unique sessile lifestyle of the nodule-bound female worm permits attack from host eosinophils, subsequent to an initial compromise of the nodular environment following loss of the *Wolbachia* endosymbiont.

10. General discussion and conclusion

10.1. The granulocyte switch leading to eosinophil activation and degranulation is preceded by *Wolbachia* depletion in oxytetracycline, but not melarsomine-treated, *O. ochengi* onchocercomata

This study sought to elucidate the temporal changes in granulocyte migration and activation in *Wolbachia*-depleted *O. ochengi*, and identify some of the mechanisms by which these changes occur. The connections between the sustained depletion of *Wolbachia* and parasite death (Gilbert et al., 2005), and the association between *Wolbachia* depletion, neutrophil decline and eosinophil accumulation (Nfon et al., 2006) had already been established, and provided a platform upon which to advance via our experiments.

The identification of a macrofilaricidal drug that did not show a direct effect on *Wolbachia* was essential for use as a 'positive control'. In a previous report, the involvement of eosinophils in the killing of *Wolbachia*-depleted worms could only be inferred because a non-antibiotic macrofilaricidal drug was not used in parallel (Nfon et al., 2006). Thus, our study included a melarsomine-treated group, which was necessary to compare the role of eosinophils between worms depleted of *Wolbachia* and those killed by a direct mechanism. Tchakouté et al (2006) reported the anti-filarial activity of melarsomine in *O. ochengi* onchocercomata, but the specific effect on *Wolbachia* was not examined. Our data using the naturally *Wolbachia*-infected *Aedes albopictus* mosquito cell line unequivocally demonstrated that melarsomine exerted no direct effect on *Wolbachia* within this system. The results showed that *Wolbachia* numbers continued to increase, despite some

evidence of host cell inhibition during melarsomine treatment, in contrast to early *Wolbachia* decline in the doxycycline-treated group.

Accordingly, similar non-deleterious effects on filarial *Wolbachia* were evident during the analysis of *O. ochengi* onchocercomata treated with melarsomine. Quantitative RT-PCR analysis of *O. ochengi* and *Wolbachia* mRNA transcripts revealed a reduction in *Wolbachia* density only subsequent to worm degeneration in the melarsomine-treated group. This was in contrast to an earlier reduction in *Wolbachia* numbers in the oxytetracycline-treated group.

Analysis of the temporal effects of melarsomine on filarial versus *Wolbachia* structures was an important consideration, because arsenicals can exhibit both antibacterial (Riethmiller, 2005) and antifilarial (Dickerson and Thompson, 1966) properties. In spirochaetes (and trypanosomes), arsenicals interrupt metabolism by binding with sulfhydryl groups within metabolic enzyme pathways (Eagle, 1939; Voegtlin, 1925). Their mechanism of action is thought to be similarly linked to metabolic inhibition in filariae (Bhargava et al., 1983) and indeed, in mammalian tissue (Voegtlin, 1925). Thus, a toxic effect on *Wolbachia* exposed after the lysis of host mosquito cells within a cell culture system could be expected. To ensure the gradual changes within treated Aa23 cell cultures were measurable, careful consideration was also given to the dose of melarsomine used, in order to avoid simultaneous obliteration of all cells. Our results showed that melarsomine had no direct toxic activity against *Wolbachia*.

The inclusion of melarsomine in the *in-vivo* experiment enabled the comparison of spatial factors relating granulocyte infiltration, worm viability and *Wolbachia* load. In the negative control group, neutrophils consistently outnumbered eosinophils and

as expected, the ratio did not change significantly. However, in the oxytetracycline-treated group, neutrophils started to decline and eosinophils infiltrated as early as 8 wpt; moreover, they were seen to degranulate extensively in the vicinity of viable, reproductively active worms. The maximum eosinophilic numbers were sustained between 4 and 48 wpt and the level of degranulation was much more intense than at any time in the melarsomine group. At 12 wpt, the median eosinophil degranulation score peaked, and was accompanied by severe ultrastructural (and probably irreversible) abnormalities in the worms in the oxytetracycline-treated group. A complete rupture of the cuticle was not observed via electron microscopy at 12 wpt, but a breach in the parasite defence was evident, as eosinophils were found in the pseudocoelomic cavity. Although a sectioning artefact cannot be ruled out, it appears very unlikely because there was no evidence of tissue dragging or a more general loss of local structural integrity of the worm. More likely, it represented an occurrence of diapedesis through stomas such as the vulva, which could have occurred as a result of sustained damage to the worms' normal barrier function.

An important observation was that lower levels of eosinophil degranulation were apparent accompanying worms that showed signs of damage, which strongly suggested that eosinophils migrated early and became activated as a result of *Wolbachia* loss rather than worm morbidity. Although an eosinophil influx was observed in the melarsomine group, it was not statistically significant and occurred very late in comparison with the oxytetracycline group. In addition, the late influx was accompanied by macrophages and was coordinated with worm fragment clearance and nodule resolution; thus, this was more consistent with the process of wound healing. This is not surprising, given the reported role of eosinophils in tissue remodelling and wound repair. Studies using hamster skin identified eosinophils as

important contributors of cytokines such as TGF-α and β1 to the wound healing milieu (Wong et al., 1993). The secretion of TGF-β1 from eosinophils, as well as classical Th-2 cytokines such as IL-4 and IL-13, stimulated fibroblast activation and collagen synthesis in human atopic tissue (Phipps et al., 2002), in accordance with a wound healing setting.

The mode of action of other antifilarial drugs has been posited to be wholly or partially dependent on the host immune system. The macrocyclic lactone ivermectin exerts a relatively fast filaricidal action on the larval stages *in-vivo* (Renz et al., 1995). Ivermectin disrupts neurotransmission by binding to glutamate-gated chloride (GluCl) channels (Wolstenholme and Rogers, 2005), but the specific effect on filarial parasites is unclear. However, it is suggested that the mammalian immune system contributes towards microfilarial death in ivermectin-treated individuals, because *in-vitro,* a much higher concentration of ivermectin is required to cause comparable microfilarial damage (Bennett et al., 1988). In support of this, it has recently been demonstrated that ivermectin, via the inhibition of GluCl channels, disrupts the release of excretory-secretory proteins that may normally serve to modulate the host immune system (Moreno et al., 2010). These findings concord with earlier observations in ivermectin-treated patients, where damaged microfilariae collecting in the regional lymph nodes were vulnerable to host immune attack, and were surrounded by degranulating eosinophils (Wildenburg et al., 1994). Eosinophils were also observed attacking damaged microfilariae in *O. volvulus* nodules following ivermectin chemotherapy, alongside an increase of other immune effector cells such as mast cells, neutrophils and macrophages (Wildenburg et al., 1998). In addition, neutrophils were found to be activated after ivermectin treatment, indicated by elevated plasma elastase levels in treated patients (Njoo et al., 1993).

Enhanced attachment of eosinophils and neutrophils to damaged microfilariae was also reported in the skin after treatment with DEC (Gutierrez-Pena et al., 1996), although *in-vitro* evidence supports a greater role for eosinophils (Medina-De la Garza et al., 1990). DEC requires components from the host immune system to cause microfilarial death, as the pre-treatment of eosinophils and not microfilariae with DEC increased eosinophil adherence rates towards this stage *in-vitro* (Medina-De la Garza et al., 1990). However, DEC is also thought to cause minor damage to microfilariae *per se* by inducing changes in their surface layers (Piessens and Beldekas, 1979).

Antibiotics have also been employed in the last decade for the treatment of filariases. The bacteriostatic effects of the tetracyclines have been known for more than seven decades [reviewed in Chopra and Roberts (2001)]. Tetracyclines inhibit protein biosynthesis within the bacterial cell by binding to the 30S subunit of the bacterial ribosome. The bacteriostatic mechanism is also effective against *Wolbachia* (Hermans et al., 2001), thus exerting an indirect negative effect on filarial vitality (Townson et al., 2006) that eventually results in worm death (Gilbert et al., 2005; Langworthy et al., 2000). Gilbert et al. (2005) demonstrated that a protracted, intermittent course of oxytetracycline was required to sufficiently reduce the *Wolbachia* load below a threshold required for *O. ochengi* worm survival *in-vivo*. In their study, only worms treated with a short intensive two-week course of oxytetracycline recovered in accordance with *Wolbachia* recrudescence, while worms treated for 2 weeks daily, then monthly for 5 months, decreased in metabolic activity and were dead by 9 months post-treatment (Gilbert et al., 2005). The *Wolbachia* threshold concept is consistent with our *ex-vivo* nodule transcript data, which revealed that in oxytetracycline-treated nodules at 12 wpt, *Wolbachia* 16S

rRNA dropped to a level which was lower than *Oo*GST (the marker used to determine worm viability). From this point forward, *Oo*GST levels declined rapidly, indicating that the level of *Wolbachia* had dropped below a threshold that was compatible with worm viability. A similar pattern appears to be apparent in the treatment of *O. volvulus*, as a long (6-week) course of doxycycline is required to attain macrofilaricidal effects (Hoerauf et al., 2008).

A contributory role of *Wolbachia* to the normal metabolism of the worm is a well-supported hypothesis, since the depletion of *Wolbachia* coincides with a decrease in worm vitality. The observation that filarial motility declined post-tetracycline treatment concurrently with a decrease in *Wolbachia* suggests that *Wolbachia* may contribute directly to the metabolic activity of the worm (Dangi et al., 2009; Townson et al., 2006). Dangi et al. also revealed that proteins important for cellular cytoskeleton integrity, and mechanisms countering stress were down-regulated in *Wolbachia*-treated worms (Dangi et al., 2009). Furthermore, gene transcription for proteins implicated in metabolic pathways and cuticular formation were down-regulated in tetracycline-treated *B. malayi* (Foster et al., 2005; Ghedin et al., 2009). In contrast, Strübing et al (2010) showed that *Wolbachia*-depleted *B. malayi* up-regulated haem-binding proteins and subunits of respiratory chain complexes containing haem and riboflavin, presumably in an attempt to maintain respiratory homeostasis (Strubing et al., 2010). Thus, antibiotic treatment could impede any nutritional symbiosis afforded by *Wolbachia* (Foster et al., 2005). In this case, our results may indicate that the host immune effector cells play a role in the destruction of worms that are compromised via slow starvation of their nutritional dependencies.

Nfon proposed that in *O. ochengi*, worm damage following *Wolbachia* depletion involves the host immune system, as eosinophils infiltrated the nodule following *Wolbachia* depletion, but receded following *Wolbachia* recrudescence (Nfon et al., 2006). Doxycycline used at 100 times the minimum bactericidal concentration for *Wolbachia* [as determined in the Aa23 mosquito cell line (Hermans et al., 2001)], was reported to elicit significant antifilarial activity against *O. gutturosa in-vitro* (Townson et al., 2006). However, in the same study oxytetracycline exerted no detectable activity against *O. gutturosa* (Townson et al., 2006). With our data using oxytetracycline, it is clearly apparent that the mammalian immune system contributes to the demise of *Wolbachia*-depleted *O. ochengi*. Indeed, our cellular histology data are consistent with Nfon et al (2006), and in addition we confirmed the active involvement of eosinophils in mediating structural damage of *O. ochengi* subsequent to *Wolbachia* depletion.

10.2. The contribution of our data in the elucidation of cellular changes within the oxytetracycline-treated *O. ochengi* nodule

Ex-vivo quantification of a selection of cytokines known to act upon granulocytes, together with *in-vitro* bovine granulocyte cell migration assays, were performed to further understand the mechanisms behind the cellular milieu inside untreated nodules. The longitudinal nature of the *in-vivo* experiment, together with the high nodule load and ease of extirpation, allowed the removal of two nodules at each time point. Thus, the histological and cytokine parameters from the same animal at a specific time-point could be evaluated together, to gain an overall insight into the temporal changes during and after treatment. This is a particularly powerful application of the *O. ochengi* model.

To determine the relationship between the presence of *Wolbachia* and the infiltration of neutrophils, we collected *ex-vivo* transcript and histopathology data, and also performed *in-vitro* cell migration assays. Our *ex-vivo* cytokine data revealed that IL-8 and GRO production in the nodule was related to the presence of *Wolbachia*, or *Wolbachia* products, because transcripts for both chemokines decreased significantly in parallel with *Wolbachia* depletion in the oxytetracycline-treated group. This was in contrast to no significant change in the melarsomine or control groups. The data corroborates previous reports implicating *Wolbachia* in the up-regulation of neutrophilic cytokines. Recombinant WSP from *D. immitis* was shown to increase production of IL-8 when incubated with neutrophils from healthy dogs (Bazzocchi et al., 2003). Similarly, IL-8 and TNF-α were produced from monocytes and mononuclear cells from healthy human donors when incubated with *Wolbachia*-replete *O. volvulus* extracts, compared to *Wolbachia*-depleted worms (Brattig et al., 2001). A strong up-regulation of IL-8 was also observed upon incubation with untreated *O. ochengi* worm extracts (Brattig et al., 2001). In the *B. malayi* mouse model, *in-vitro* incubation studies revealed that the production of TNF-α and the chemokines MIP and KC from neutrophils increased significantly in the presence of *Wolbachia*-replete worm extracts, in contrast to *Wolbachia*-depleted worms (Gillette-Ferguson et al., 2004).

Although the attraction of neutrophils towards *Wolbachia* (or *Wolbachia* products) using cell migration assays has been previously demonstrated in other species, our data are the first of *O. ochengi*. Consistent reports from King et al (1983) and Rubio de Kromer et al. (1998) confirmed that neutrophils were attracted towards *O. volvulus* microfilariae (King et al., 1983) and extracts of female macrofilariae *in-vitro* (Rubio de Kromer et al., 1998). At this early stage, Rubio de Kromer et al.

(1998) proposed that one of the chemoattractive proteins separated by Western blot belonged to *Wolbachia*. Later studies using *Wolbachia*-depleted *O. volvulus* extracts (Brattig et al., 2001), or recombinant *Wolbachia* surface protein from *D. immitis* (Bazzocchi et al., 2003), confirmed that *Wolbachia* or its products were indeed chemoattractive for neutrophils. Neutrophils were found to migrate towards *O. volvulus* extracts containing *Wolbachia*, but did not migrate towards worms depleted of *Wolbachia* via antibiotic chemotherapy (Brattig et al., 2001).

Results from our cell migration studies showed some comparable trends with those from previous publications. Our neutrophil migration data exhibited an average chemotactic index of ~3 for untreated male worms, and ~1.6 for untreated female worms, which was comparable to a chemotactic index of ~2 for female worms at an antigen concentration of 10 $\mu g\ ml^{-1}$ in previous studies (Brattig et al., 2001; Rubio de Kromer et al., 1998). In addition, our data showed that there was a ~50% decrease in neutrophil migration towards *Wolbachia*-depleted female worm extracts which, although not statistically significant, is also consistent with data from Brattig et al (2001). However, no such trend was apparent between the *Wolbachia*-replete and –depleted male worm extracts. Moreover, the naturally aposymbiotic filaria, *S. labiatopapillosa*, induced neutrophil chemoattraction to a similar degree to that observed with *Wolbachia*-positive *O. ochengi*. Although male *O. ochengi* extracts were not included in previous studies, and therefore no direct comparison can be made with regard to *in-vitro* migration assays, neutrophils have been observed in the vicinity of male worms in the nodule (Brattig et al., 2001).

The reasons for the inconsistency between our results and those of previously published studies are probably due to differences in antigen preparation. To obtain

Wolbachia-depleted material for the *in-vitro* assays, animals were treated with a short intensive regimen of oxytetracycline. This was implemented to ensure that the recovered worms were still viable, despite their reduced complement of *Wolbachia* (Gilbert et al., 2005). Although this was an important precaution to exclude the effects of general worm degeneration, *Wolbachia* levels may not have been as low as was the case in the *ex-vivo* nodules after prolonged chemotherapy. Therefore, it is possible that the treated worm antigen retained sufficient *Wolbachia* products to attract neutrophils *in vitro*. Importantly, in the study of Brattig et al. (2001), the female worm extracts were obtained from nodules removed at least 4 months after a 6-week doxycycline regimen, and thus residual *Wolbachia* levels may have been lower than in our *in vitro* assays. With regard to the possible differences between the responses to the male and female worm extracts, oocytes and embryos have been shown to be more vulnerable to the elimination of *Wolbachia* by tetracyclines than is somatic tissue (Ghedin et al., 2009). These authors and others have also hypothesised that worm gene expression following *Wolbachia* depletion may be affected differently between male worms and the female reproductive tract (Ghedin et al., 2009; Heider et al., 2006). Thus, the differential effects of oxytetracycline on the depletion of *Wolbachia* between male and female worms may be reflected in a difference in their neutrophil chemotactic potency. However, perhaps the most important consideration is that a direct comparison between the *in-vitro* system and the actual events occurring within onchocercomata *in vivo* may not be valid due to substantial temporal differences. Changes in cell populations that occur over weeks or months in response to a gradual reduction in endobacterial density simply cannot be recreated in the laboratory.

Taken together, our data support the evidence from other filarial models that *Wolbachia* induces the up-regulation of neutrophilic cytokines, and drives the infiltration of neutrophils. However, although *Wolbachia* share components in common with other bacteria that are known to induce a pro-inflammatory neutrophilic response, their intracellular distribution within the filarial host would seem to deny direct access to the mammalian immune system.

Wolbachia resides *within* filarial cells which in turn reside within the mammalian host; a 'Russian doll' effect. Despite this, there is evidence to show that mammalian immune cells do indeed come into contact with, and even phagocytose, *Wolbachia*. *Wolbachia* surface protein was detected in the phagosomes of activated neutrophils within the murine cornea as demonstrated by immunoelectron microscopy (Gillette-Ferguson et al., 2004). WSP was also detected in activated macrophages that were observed surrounding degenerating *O. volvulus* in nodules from patients treated with suramin (Brattig et al., 2004a).

The death of microfilariae after treatment with relatively rapidly-acting filaricidal drugs, in particular in heavily-infected patients, is followed by an immense release of *Wolbachia*, and this represents a significant challenge to the host immune system (Abasht et al., 2008; Cross et al., 2001). In DEC-treated lymphatic filariasis patients, the severe systemic reactions that were observed post-treatment were positively correlated with *Wolbachia*-positive blood samples (Cross et al., 2001). A similar situation was evident in *O. volvulus* patients, as *Wolbachia* DNA was detected in the serum of DEC and ivermectin-treated patients (Keiser et al., 2002). In the ivermectin treated patients, an increase in TNF-α in correlation with *Wolbachia* DNA was reported, and peak DNA levels corresponded with the highest scores of inflammation

(Keiser et al., 2002). The release of *Wolbachia* DNA into the circulatory system also occurs in *O. ochengi*-infected cattle (Nfon, Trees & Makepeace, unpublished data).

Even in untreated individuals, there is evidence that *Wolbachia* represents a continuous antigenic stimulus. Since patients in hyperendemic areas of *O. volvulus* may experience microfilarial turnovers of up to 300,000 per day (Duke, 1993), the continued presentation of *Wolbachia* to host immune cells is inevitable. In addition to the release after natural worm attrition (Brattig et al., 2004b), the presence of *Wolbachia* within uterine fluid from female worms during parturition is highly likely (Schulz-Key, 1988; Taylor et al., 2001). Recently, *Wolbachia* surface protein has been identified independently of intact bacteria throughout *B. malayi* somatic tissues, including the cuticle, eggshells and within the pseudocoelomic fluid (Melnikow et al., 2011). Furthermore, *Wolbachia* proteins have been detected in the excretory/secretory products of cultured worms (Bennuru et al., 2009), and *Wolbachia* cells have been observed in the secretory canal lumen of *B. malayi* (Landmann et al., 2010). Taken together, the evidence suggests that despite its residence within the 'protective' confines of the filarial host, *Wolbachia* is continuously accessible to the mammalian immune system.

Accordingly, *Wolbachia* (or its products released into the host environment) are recognised by the mammalian immune system. *Wolbachia*-derived molecules such as WSP (and potentially GroEL) are recognised as pathogen-associate molecular patterns (PAMPs) via pathogen recognition receptors, principally Toll-like receptors, on host cells including macrophages and PMNs (Rollinson and Hay, 2008). Both TLR-2 and TLR-4 were required in the recognition of WSP by murine macrophages and dendritic cells incubated with *D. immitis* rWSP (Brattig et al., 2004b).

Furthermore, WSP was implicated in the migration of neutrophils into the murine cornea (Gillette-Ferguson et al., 2004), and their recruitment and activation was mediated by corneal cells that produced neutrophilic chemokines upon stimulation of TLR-2 (Gillette-Ferguson et al., 2007). GroEL has been identified as a potential PAMP, as in lymphatic filariasis, the heat-shock protein was associated with the upregulation of IgG in patients expressing chronic pathology (Suba et al., 2007). *Wolbachia*-derived diacylated lipoprotein (also known as peptidoglycan-associated lipoprotein, PAL) was also found to induce host inflammatory responses via the activation of TLR-2 and -6 in *B. malayi* infection in mice (Turner et al., 2009).

There is some overlap in TLR expression on the surface of eosinophils and neutrophils (Nagase et al., 2003), which is unsurprising since neutrophils express most (TLR-3 is a notable exception) of the TLRs identified to date (Hayashi et al., 2003). However, the recognition of bacterial molecules such as diacylated lipoproteins (effects previously ascribed to peptidoglycan due to contamination) by TLR-2 on neutrophils but not eosinophils (Mattsson et al., 2003) concurs with evidence from studies on the recognition of *Wolbachia* products by TLRs (Brattig et al., 2004b; Gillette-Ferguson et al., 2007; Turner et al., 2009). Thus, it is certainly feasible that in the nodule, *Wolbachia* play a role in the recruitment of neutrophils via TLR recognition.

Following the demonstration of a significant association between *Wolbachia* and the existence of a neutrophilic environment within *O. ochengi* nodules, we sought to identify the cytokine pathways that may facilitate the neutrophilic milieu. The *ex-vivo* transcript data suggested that neutrophils may be a key source of IL-8 and GRO, because the reduction in neutrophilic chemokine transcripts followed the decrease in

neutrophils in oxytetracycline-treated nodules. Thus, the decrease in these chemokines was probably an effect of, rather than a cause of, the decline in neutrophil numbers. This agrees with data from Bazzocchi et al., who reported that WSP stimulated IL-8 production from canine neutrophils (Bazzocchi et al., 2003).

It would follow, then, that a reduction of neutrophils would lead to a reduction in IL-8 within the nodule. However, because the decline in neutrophils was not preceded by a decrease in IL-8 or GRO transcripts, the mechanisms of initial neutrophil infiltration remain to be elucidated. Indeed, if IL-8 and GRO are involved in this process, then the initial source would presumably be a cell population other than neutrophils. This is entirely feasible, as both cytokines are produced by other cells, in particular macrophages and dendritic cells.

Macrophages have been implicated in the granulomatous response during early nodule formation of *O. volvulus* (Brattig, 2004a), and may even contribute to neovascularisation in the developing nodule (Attout et al., 2009). Macrophages also reside in relatively high quantities in untreated *O. ochengi* nodules (Nfon et al., 2006). Furthermore, IL-8 (together with TNF-α) was produced from blood monocytes in response to *Wolbachia*-replete *O. volvulus* and also *O. ochengi* (Brattig et al., 2001). Therefore, it is possible that macrophages are indirectly responsible for the initial influx of neutrophils into the developing nodule via a positive feedback loop, especially as *Wolbachia* products have been implicated in the up-regulation of TNF-α (Brattig et al., 2001), and in turn, TNF-α can up-regulate GRO production in macrophages (Becker et al., 1994).

The neutrophilic environment within the normal onchocercoma may involve other cytokines and intermediary effector cells, or may even result from a direct

chemotactic effect from *Wolbachia* products. ENA-78 is a potent neutrophilic chemoattractant produced by epithelial cells, monocytes and macrophages (Allmann-Iselin et al., 1994), in addition to eosinophils (Persson et al., 2003) and mast cells (Lukacs et al., 1998). ENA-78 represents an important candidate for further investigation, particularly because eosinophils (and a small number of mast cells) were observed scattered around the periphery of the neutrophilic infiltrate in control nodules. ENA-78 (Imaizumi et al., 1997), together with IL-8 and GRO (Shaik et al., 2009), is reported to be secreted from vascular endothelium, which may be significant since the normal onchocercoma is also highly vascularised (Smith et al., 1988).

Because of the recruitment of macrophages and neutrophils into the untreated onchocercoma, *Wolbachia* have been implicated in the initiation of a Th-1 response (Brattig, 2004a; Hoerauf et al., 2003b). Furthermore, Th-1 and Th-17 cells have been implicated in the progression of lymphatic pathology in bancroftian filariasis (Babu et al., 2009). Th-17 cells have also been suggested to contribute to immune-mediated pathology following *Wolbachia* PAL-mediated up-regulation of proinflammatory cytokines by dendritic cells (Turner et al., 2009). If this is indeed the case, the production of Th-17 cytokines may in turn up-regulate the production of IL-8 from macrophages (Gu et al., 2008); and thus *Wolbachia* may indirectly drive neutrophil recruitment into the *Onchocerca* nodule via a potently proinflammatory process.

Thus far, our data have revealed some of the underlying mechanisms of the typical cellularity of the normal *Onchocerca* nodule. Using our *ex-vivo* histopathology and transcript data, and the *in-vitro* cell migration assays with *Wolbachia*-depleted worm extracts, we attempted to elucidate the immunological basis of the cellular switch in

antibiotic-treated nodules. Because an influx of eosinophils is related to *Wolbachia* depletion in onchocercomata, we thought it relevant to test a purified eosinophil population within the *in-vitro* cell migration chamber system. We expected to observe an increase in eosinophil migration towards *Wolbachia*- depleted worm extracts, in parallel with our observations of *ex-vivo* events. No such consistent trends were observed, as eosinophil migration remained at an index of approximately 2, however, chemotaxis of eosinophils to *Wolbachia*-negative female worm extracts tended to be greater than that for neutrophils, which was not the case for *Wolbachia*-positive antigen.

Similarly, the *ex-vivo* analysis of IL-5 and eotaxin transcripts revealed no significant relationships between the influx of eosinophils and the levels of these cytokines. The completely novel nature of this aspect of the work for a nodular *Onchocerca* spp. prevents direct data comparisons. However, our results do illustrate that the cellular switch following the depletion of *Wolbachia* is a complex sequence of events, probably involving a symphony of immune signals that may involve a combination of direct activation of mammalian cells by *Wolbachia* and filarial PAMPs, together with autocrine and paracrine cytokine networks.

An incomplete representation of the nodule environment is the most likely reason for the lack of reproducibility of *in-vivo* events in the cell migration assays. If the gradual loss of *Wolbachia* is accompanied by a gradual decline in downstream chemokine signals *in-vivo*, eosinophil infiltration may take effect over weeks, so an equivalent influx of eosinophils would not be seen during *in-vitro* assays. In relation to the eosinophil cytokine quantification, the influx of eosinophils may be dependent on the reduction of neutrophils, *i.e.*, a declining neutrophil population is a

prerequisite for the eosinophil infiltration. If this were the case, then eosinophil infiltration into the nodule may not then be associated with an up-regulation of eosinophilic cytokines, and eosinophil migration may not occur without a prior signal indicating neutrophil decline. A limitation of *ex-vivo* cytokine profiling using mRNA transcripts is that only the precursors are quantified, rather than the product itself. Moreover, since preformed cytokines are present in granulocyte granules, including IL-8 in neutrophils (Pellme et al., 2006) and IL-5 in eosinophils (Dubucquoi et al., 1994), the difference between mRNA transcript quantification and the presence of cytokines in the nodule could be considerable. Thus, quantification of the protein end-products would be preferable and may give a more accurate insight into the actual presence of cytokines at a certain point in time; however the proteomic analysis of *ex-vivo* material was not within the scope of this study.

A major enigma with regard to the cellular composition of onchocercomata is the conspicuous absence of eosinophils. Eosinophils are traditionally associated with parasitic helminth infections, and have been shown to migrate towards chemoattractants produced by these pathogens. *In-vitro* chemotaxis assays were used to demonstrate the migration of ovine eosinophils towards *T. circumcinta* and *H. contortus* E/S products (Wildblood et al., 2005), and bovine eosinophils towards an *Ostertagia ostertagi* secreted lectin (Klesius, 1993). Guinea pig eosinophils were shown to migrate towards homogenates of various parasites including *A. suum*, and *Fasciola* spp., but interestingly, not *D. immitis* (Horii et al., 1988).

Filarial worms possess or excrete molecules that have been reported to provide chemotactic signals for immune effector cells. For example, the cytokine macrophage migration inhibitory factor, discovered in the E/S products of *B. malayi*,

was found to inhibit the random migration of monocytes and macrophages in co-culture, but induced migration for these same cell types when located at a distance (Pastrana et al., 1998). Chitin was found to induce the accumulation of eosinophils (and also basophils) in murine lungs post aspiration, and into the peritoneum post-injection (Reese et al., 2007). However, in another study chitin was found to stimulate IL-17 production from macrophages, and cause tissue inflammation via pathways utilising TLR-2 and MyD88 (Da Silva et al., 2008). These apparently inconsistent results were attributed to proposed changes in the temporal effect of chitin; *i.e.*, inducing a Th-17 phenotype in acute stages, but possibly switching to a Th-2 phenotype during chronic infection (Da Silva et al., 2008). Chitin is an important constituent of the eggshells of *O. volvulus* (Brydon et al., 1987); thus, it could constitute a chemoattractant for mammalian host eosinophils, or even neutrophils. However, the adult worm cuticle is devoid of chitin, and furthermore, it has been shown to produce chitinase (Gooday et al., 1988), which is thought to aid egg formation and hatching. Nevertheless, the degradation of chitin via filarial-derived chitinase may serve to suppress eosinophilic chemotactic signals, since chitinase reduced eosinophil migration *in-vivo* (Reese et al., 2007) and *in-vitro* (Stein et al., 2009).

Eosinophils have been implicated in the destruction of helminths *in-vitro*, such as *S. mansoni* schistosomulae (Butterworth et al., 1975) and juvenile *F. hepatica* (Duffus et al., 1980). Eosinophils were suggested as important effector cells in the killing of *O. volvulus* microfilariae: in the untreated nodule (Wildenburg et al., 1996), after ivermectin treatment (Korten et al., 1998), or even after immunisation with irradiated L3 (Abraham et al., 2004). Electron microscopy of the destruction of DEC-treated microfilariae elegantly captured eosinophil-parasite adherence and the subsequent

degranulation of cytotoxic products (Racz et al., 1982). Furthermore, MBP (Kephart et al., 1984), ECP (Gutierrez-Pena et al., 1998) and MPO (Gutierrez-Pena et al., 1996) have all been implicated in microfilarial destruction following DEC or amocarzine treatment.

The role of eosinophils in the killing of adult parasites is contentious, especially in natural infections (Meeusen and Balic, 2000). However, our electron microscopy data indicated that eosinophils do contribute to worm pathology via degranulation onto the filarial cuticle, but this was only ever observed in the oxytetracycline group. Thus, the question remains as to the reason why, despite their presence in the periphery of the untreated filarial nodule, eosinophils appear to remain quiescent until the depletion of *Wolbachia* and the decline of neutrophils?

As the reduction in neutrophils in the *O. ochengi* nodule occurs around the same time as the start of the eosinophil influx, perhaps the neutrophils play a role in the marginalisation of eosinophils in the untreated nodule. Neutrophil products may contribute towards the inhibition of eosinophil migration. Neutrophilic defensins are present within the cysts formed around the anterior ends of female worms and around male worms within the nodule (Brattig et al., 2001). The antimicrobial peptide, cathelicidin, is present in the Splendore-Hoeppli deposits surrounding much of the *O. ochengi* cuticle (Armstrong, Wastling & Makepeace, unpublished data). However, published evidence indicates that these neutrophil products do not suppress eosinophil migration or activation. On the contrary, human cathelicidin induces the chemotaxis of both neutrophils and eosinophils (Tjabringa et al., 2006). There is, however, a report suggesting that small amounts of eosinophil EPO, when bound to neutrophils cause their aggregation due to increased adhesiveness between

each other and the endothelium. Furthermore, the same study reported that binding of EPO to neutrophils inhibited its catalytic activity, which was thought to occur as a protective negative feedback mechanism (Zabucchi et al., 1990). Perhaps the low numbers of eosinophils that are observed within untreated onchocercomata may contribute similar regulatory factors to aid in the aggregation of neutrophils towards the centre of the nodule.

Reciprocally, a study of the relationship between neutrophils and eosinophils in rats infected with *Mesocestoides cortii* demonstrated that peritoneal neutrophils did not increase in response to glycogen stimulus in rats that had concurrent peritoneal eosinophilia (Cook et al., 1988). These authors suggested that in infected animals, the presence of an eosinophilic response impaired the infiltration of neutrophils (Cook et al., 1988). Although similar granulocyte relationships have not been reported since, and the study could not exclude confounding effects from other host cells or the parasites themselves, it may be possible that a similar event of eosinophil-driven neutrophil inhibition occurs in the *Wolbachia*-depleted nodule.

Neutrophils may provide a physical barrier to eosinophils; however, the ability of eosinophils to diapedese through the endothelial wall (Yadav et al., 2003) renders this theory unlikely. It seems more likely that large aggregations of neutrophils may generate a cytokine 'smokescreen'. This may serve to facilitate the positive feedback loop for the recruitment of further neutrophils, and to mask any eosinophilic signals from the worm itself. One study on neutrophil chemotaxis reported that in a situation where more than one chemotactic agent is apparent, neutrophils are able to decipher their way to an end-target chemoattractant such as C5a, in priority over intermediary chemokines such as IL-8 (Heit et al., 2002). This suggests a hierarchy of

chemoattractants exists, where in a typical scenario involving multiple signals that are present simultaneously, neutrophils are able to prioritise their migratory path. This hierarchy is also present among the eosinophilic chemoattractants, where eotaxin was identified as an "endpoint" signal that had the capability of dampening the eosinophil response towards other chemoattractants (Schratl et al., 2006).

Our data helps to expose the complexity of the immune balance within the *Onchocerca* nodule, and the reason for this is probably because of the interaction of molecules from the bacteria, nematode and mammal. Thus, mammalian immune modulation could be derived from either, or both, of the two pathogens. The hypothesis of host immune modulation via filarial worm products is not new. Excretory/secretory (ES)-62, a protein secreted by filariae, was reported to skew the host immune response to a Th-2 type via the down-regulation of IFN-γ and the inhibition of IL-12 (Goodridge et al., 2001). ES-62 is also reported to be present in *B. malayi* (Bennuru et al., 2009); an abundance of which was secreted by microfilariae. The high production of ES-62 in microfilariae may explain the link between eosinophils and microfilariae observed in the nodule (Wildenburg et al., 1996). Immune modulation via *Wolbachia* products is also recognised. For instance, WSP up-regulates the expression of inducible nitric oxide synthase, which leads to an increased production of nitric oxide in mammalian macrophages (Morchon et al., 2007). Nitric oxide, although toxic for bacteria and protozoa, was found to contribute towards immune tolerance by way of directed apoptosis of antigen-specific T cells, when cultured with *B. pahangi* microfilariae (Jenson et al., 2002). Thus, in filariases, it would seem that both the symbiont and the nematode may exert a synergistic strategy for facilitating a protected environment within their mammalian host, via immune modulation.

There are four major arms of the acquired immune response that could be present in the filarial nodule: Th-1, Th-2, Th-17, and T-regulatory (T-reg) pathways. In general terms, Th1, Th2 and Th17 negatively regulate each other, and T-reg cells can suppress all of the other subsets (Belkaid et al., 2006). The main paradigm for the immune response to helminth infections is a classical Th-2 response characterised by the involvement of eosinophils, basophils, and mast cells; and an up-regulation of Th-2 cytokines including IL-4, IL-5, IL-9, IL-10 and IL-13 (Diaz and Allen, 2007). Traditionally, the Th-2 response has been considered to be a host-protective response against the parasite. Indeed, the initiation of a Th-2 response does seem essential to the clearance of gastrointestinal nematodes (McKenzie et al., 1998). In the *B. pahangi* model, IL-4 was shown to limit the fecundity of maturing worms, as IL-4 knockout mice harboured significantly more microfilariae in their peritoneal cavities than wild-type equivalents (Devaney et al., 2002). However, the manipulation of the host Th-2 response by nematodes has been proposed, as the tissue damage caused by degranulating eosinophils may be beneficial to the parasite (Wildblood et al., 2005), possibly for feeding or migration. This view has effectively been superseded by the emerging model of helminth-induced regulatory responses, which co-exist with the classical Th-2 pathway (Diaz and Allen, 2007; Gillan and Devaney, 2005). This coexistence of these pathways has been coined the "modified type 2 response", and is characterised by a decrease in IL-5 accompanied by an increase in anti-inflammatory cytokines such as IL-10 and TGF-β (Maizels and Yazdanbakhsh, 2003).

Our results indicate that, in the natural *O. ochengi* nodule, there is little evidence of a classical parasite-mediated Th-2 response. Eosinophils, basophils and mast cells are scant, and IL-5 expression is low. However there is mounting evidence to indicate a

regulatory milieu. Graham et al. showed that in *O. ochengi* infection, both Th-1 and Th-2 responses were down-regulated in synchrony with filarial maturation (Graham et al., 2001). Doetze et al reported a Th-3 response (also known as "natural" T-reg) in the mediation of the hyporesponsive form of human onchocerciasis (Doetze et al., 2000). These reports agree with Taylor et al., (2005a) who demonstrated a role for T-reg cells in the survival of *L. sigmodontis* in mice. In addition, alternatively activated macrophages are associated with patent infections of *L. sigmodontis* (Taylor et al., 2006), and their down-regulatory influence on T-cell responses is associated with enhanced parasite survival (Hoerauf and Brattig, 2002). It is not yet clear whether macrophages in the onchocercoma are classically activated or alternatively activated, or whether there is a switch in activation associated with chronicity. However, recent research reporting the up-regulation of TGF-β from macrophages and T-cells in *O. volvulus* nodules implies that alternatively activated macrophages are present in the mature onchocercoma (Korten et al., 2010).

Although not traditionally associated with helminth infections, our data indicate a Th-1 or perhaps a Th-17 environment in the nodule. The accumulation of neutrophils, together with the presence of the pro-inflammatory cytokines IL-8 and GRO, are all associated with a role in inflammation, in common with a classical response to intracellular bacteria. Furthermore, Th-17 cells, via the release of IL-17, orchestrate and amplify neutrophil function in resistance against bacteria [reviewed by Fouser et al. (2008)]. IL-17 is associated with the induction of mediators that promote granulopoiesis and consequent neutrophil proliferation and accumulation (Pelletier et al., 2010). IL-17, together with IL-8, IFN-γ, TNF-α, and granulocyte–macrophage colony-stimulating factor (GM-CSF), favour the recruitment, activation and prolonged survival of neutrophils at sites of inflammation (Pelletier et al., 2010).

A potent IL-17 phenotype would also be consistent with the intense neutrophilia that we observed in untreated onchocercomata.

In a nodule which may contain a combination of immune pathways, there may be cross-talk and interference between each. For example, filariae that secrete products that would induce a typical Th-2 response may be 'lower down' in a hierarchy of signals, and thus may be 'drowned out' by stronger signals from *Wolbachia*. A cytokine hierarchy would explain the mixed cellularity within the *B. pahangi* granuloma. In *B. pahangi*-infected jirds, a low level of IL-5 was present in granulomas, despite the presence of eosinophils close to adult worms (Rao and Klei, 2006). However, macrophages and giant cells greatly outnumbered eosinophils in these granulomas, which were also found to express IFN-γ. Furthermore, the cytokines TNF-α and IL-6 were also secreted by antigen-stimulated granulomas *in-vitro*, and this may be attributable to the influence of *Wolbachia* within the *B. pahangi* extract (Rao and Klei, 2006). Thus, in *B. pahangi* granulomas, there is evidence of a mixture of signals, although the "consensus" is thought to be Th1-weighted (Rao and Klei, 2006).

Another possibility is that *O. ochengi* may be driving a T-regulatory response within the nodule. As previously discussed, both Th-1 and Th-2 responses were down-regulated in accordance with maturation of *O. ochengi* (Graham et al., 2001). In *O. volvulus*, FOXP3+ Treg cells were found to be strongly induced by dead worms in hyporeactive nodules (Korten et al., 2008). The observation of further upregulation of these cells in doxycycline-treated nodules suggested that the causation of the T-regulatory milieu was of filarial origin and not from *Wolbachia*, and moreover indicated that *Wolbachia* may dampen this regulatory response in the normal nodule

(Korten et al., 2008). A further study revealed that in *O. volvulus* nodules, the observed up-regulation of TGF-β is consistent with a role in Th-1 and Th-2 suppression (Korten et al., 2010). However, the presence of neutrophils and macrophages close to filarial worms supports the suggestion of a Th-1 or Th-17 weighted milieu. In addition, the accumulation of defensins around adult female filariae further illustrates the localised activation of neutrophils (Brattig et al., 2001), and supports the hypothesis of a *Wolbachia*-induced Th-1/Th-17 cellular environment. Thus, although there is strong evidence to suggest an overriding T-reg milieu in the case of patent *Onchocerca* infections, this does not explain the intense neutrophilic environment found in the *Onchocerca* nodule. However, the fact that these neutrophils do not cause the localised necrosis and tissue damage normally characteristic of, for example, a staphylococcal abscess (Zhao and Lacasse, 2008), could be attributed to a regulatory influence.

In sowda patients, nodules are small, scarce, and highly eosinophilic; while adult worms are reduced in size and their numbers are low (Brattig, 2004a). Interestingly, a Th-2 response seems to dominate in these cases, since neutrophils become hyporeactive [as evidenced by a lack of response to fMLP (Rubio de Kromer et al., 1995)], and an infiltration of mast cells and eosinophils occurs, together with the production of IgE (Korten et al., 1998). An increased frequency of a polymorphism in the IL-13 gene found in sowda patients (Hoerauf et al., 2002) supports the suggestion of a switch towards a Th-2-biased response in these patients. The hyperreactive effects seen in sowda patients may represent the best natural analogue of the cellular responses in the tetracycline-treated nodule. Hence, it is very interesting that in sowda, partial clearance of infection does occur (Brattig, 2004a),

and that eosinophils have been linked to larval destruction in humans (Brattig et al., 1991) and mice (Folkard et al., 1996).

Taken together, our data provides evidence to suggest that in the *Onchocerca* nodule, a Th-1/Th-17 weighted immune response is present, and that this is associated with *Wolbachia*. Changes in cell populations consistent with a Th-2-biased response are evident with the decline of *Wolbachia*, and these events occur when the worms are still viable. Furthermore, evidence from our study supports the notion that any Th-2 stimulus from the filarial worm is overridden by a stronger *Wolbachia*/Th-1 axis. Thus, when the symbiosis breaks down following oxytetracycline chemotherapy, a classical Th-2 response ensues, leading to worm destruction via the activation and degranulation of eosinophils.

10.3. The defensive mutualist role of *Wolbachia* in the *Onchocerca* nodule - a variation on a theme?

In insects, *Wolbachia* exerts reproductive manipulative effects and seeks to maximise its own fitness, as it is vertically transmitted through the female germ-line only. For example, the sex-ratio distortion of the woodlouse *Armadillidium vulgare* towards females has been attributed to the effects of *Wolbachia*, which inserts a feminising factor into the Z heterochromosome of these arthropods (Juchault and Mocquard, 1993). *Wolbachia*-mediated male killing in the ladybird *Adalia bipunctata*, and in the butterfly *Acraea encedon*, effectively removes any competition from growing males, thus conferring superior fitness on the females in a brood (Hurst et al., 1999). In *Drosophila*, the positive correlation between *Wolbachia* density and the expression of cytoplasmic incompatibility (Bourtzis et al., 1996) ensures the perpetuation of the most successful colonising genotypes of the bacteria.

Further to their role in reproductive manipulation, *Wolbachia* has demonstrated a variety of mutualistic traits in arthropods. *Wolbachia* (strain *w*Mel) was found to confer antiviral resistance and tolerance against Drosophila C virus in both *Drosophila melanogaster* and *D. simulans,* and *Wolbachia* densities in these flies positively correlated with the level of protection (Osborne et al., 2009). In *D. melanogaster*, *Wolbachia* also provided resistance to two other RNA viruses, Nora virus and cricket paralysis virus (Hedges et al., 2008; Teixeira et al., 2008). Although the replication of Flock House virus was not inhibited as strongly in *Wolbachia*-infected flies, resistance to pathology associated with the virus was conferred (Teixeira et al., 2008). Partial resistance to infection with West Nile virus was also observed in the naturally *Wolbachia*-infected mosquito, *Culex quinquefasciatus*, in addition to *D. melanogaster* (Glaser and Meola, 2010). The resistance was abated in flies depleted of *Wolbachia* via tetracycline treatment.

In *Asobara tabida* wasps, *Wolbachia*-depleted females from infected populations either could not produce any eggs, or if they did, the larvae died soon after hatching (Dedeine et al., 2005). Moreover, in the springtail *Folsomia candida*, *Wolbachia* was found to be essential for egg hatching, as individuals that were depleted of *Wolbachia* did lay clutches of eggs, but all were unviable (Timmermans and Ellers, 2009). Interestingly, in the same study the author discussed the observation that there seemed to be a critical threshold of *Wolbachia*, below which the springtails were unable to reproduce (Timmermans and Ellers, 2009). In perhaps the most subtle *Wolbachia* symbiosis described in arthropods to date, a recent report described an obligate mutualistic dependency in *D. paulistorum*, manifested by low female fecundity and male-biased sex-ratio distortions in *Wolbachia*-cured flies (Miller et al., 2010). Moreover, *Wolbachia* depletion was found to influence female mating

behaviour, triggering mate recognition mechanisms that led to avoidance of male flies carrying incompatible *Wolbachia* variants (Miller et al., 2010). Evidence is also emerging to support the role of *Wolbachia* as a nutritional mutualist in arthropods. *Wolbachia* was shown to provide the common bedbug *Cimex lectularius* with B-vitamins, as elimination of *Wolbachia* induced growth retardation and sterility in the host, both of which were compensated by the addition of oral B vitamin supplements (Hosokawa et al., 2010).

Wolbachia also appears to be vital in the contribution of nutritional factors in filariae. For instance, the genomic sequencing of *B. malayi* indicates (via absence of genes for specific metabolic pathways in each partner) that *Wolbachia* may provide the filarial host with riboflavin, haem and purines (Ghedin et al., 2007), in exchange for the amino acids biotin and folate required by the endobacterium (Foster et al., 2005). The proposed nutritional dependencies of the host worm is certainly compatible with observations from previous studies of *O. ochengi* that demonstrated continued worm viability, but reduced motility, for some months after antibiotic-mediated depletion of *Wolbachia* (Gilbert et al., 2005; Langworthy et al., 2000). These observations may be consistent with chronic malnutrition associated with the depletion of *Wolbachia* below a critical threshold, but they do not explain the occurrence of activated eosinophils degranulating on viable worms, as was found in our study. However, these studies do challenge the hypothesis that the death of *Wolbachia* may cause a toxic effect on the worms, as a transient depletion of *Wolbachia* did not kill or permanently sterilise adult *O. ochengi* (Gilbert et al., 2005).

In arthropod hosts, there is also evidence that the defensive mutualism phenotype may be generated by manipulation of the innate immune system. In an example of an unnatural *Wolbachia* infection, *Aedes aegypti* mosquitoes experimentally infected with the *w*MelPop strain showed a significant decrease in susceptibility to infection with *B. pahangi*, as evidenced by a reduction in filarial prevalence and intensity following challenge with microfilariae (Kambris et al., 2009). Intriguingly, *w*MelPop also conferred increased resistance to infection with *Erwinia carotovora*, a Gram-negative bacterium which killed the *Wolbachia*-negative strain, and an up-regulation of immune factors, including two forms of the antimicrobial peptide cecropin (Kambris et al., 2009). Further studies using *Aedes aegypti* co-infected with *Wolbachia* and dengue virus showed that *Wolbachia* excluded dengue virus from ommatidia cells of the eye, and also brain and fat tissue within the mosquitoes. In addition, these mosquitoes did not secrete dengue virus in their saliva, and viral protein synthesis was markedly reduced (Moreira et al., 2009).

Although the evidence for the role of *Wolbachia* as a defensive mutualist in arthropods is growing, the elucidation of the role of *Wolbachia* in filariae must take into account its relationship not just with the filarial host, but also a third party, the mammalian host. This 'Russian doll' scenario is also apparent in some arthropod species however, and thus parallels can be drawn with the immunomodulation of the third-party host. For instance, *Wolbachia* infecting the larvae of the leaf-mining moth *Phyllonorycter blancardella* manipulates the cytokinin levels of the plant host to produce photosynthetically-active green patches on otherwise senescent leaves, thus providing food for the larvae during autumn (Kaiser et al., 2010). Similarly, *Wolbachia* within larvae of the root-feeding beetle *Diabrotica virgifera virgifera*

causes the down-regulation of maize defence genes, including chitinase, defensins and lignin, when infected larvae feed on the plant roots (Barr et al., 2010).

Given the massive diversity and complexity of the effects of *Wolbachia* between species, added to the various immune challenges presented at each stages of the filarial lifecycle, it would be short-sighted to assume that *Wolbachia* confers the same immunomodulatory effect throughout the worms' life. Indeed, in the remarkable partnership between the entomopathogenic nematodes and their endosymbionts, each key lifecycle event is exemplified by different mutualistic interactions that elegantly ensure the optimum outcome for the symbiotic partners. For example, the insect parasitoid *Steinernema* and its endosymbiont *Xenorhabdus* work together to subvert the insect immune system, via mechanisms such as the suppression of melanisation via the liberation of LPS. After insect death, *Xenorhabdus* bioconverts the contents of the cadaver to furnish the nematode with the nutrients needed for growth and reproduction, concomitantly preserving the cadaver via the production of antimicrobial compounds. This ensures that maximum nutritional resources are available to its future vehicles; *i.e.*, the brood of juvenile *Steinernema* (Koppenhöfer and Gaugler, 2009).

In contrast to the intricate studies on entomopathogenic nematodes, little is known about the effects of *Wolbachia* during the larval stages of filarial infection. However, a recent publication on *L. sigmodontis* L3 reported that *Wolbachia* may modulate the host immune system to generate an environment that facilitates initial larval entry and migration. *Wolbachia* was found to mediate the recruitment of mast cells, which in turn increased vascular permeability via histamine release (Specht et al., 2011). Instillation of cromolyn to inhibit mast cell release of histamine via degranulation

effectively resulted in a decrease in the filarial load. Furthermore, mast cells were not activated in infections using *Wolbachia*-depleted worms (Specht et al., 2011). Importantly, the same study identified CCL-17 as a potentially protective chemokine, and this molecule was reported to be produced by T-cells in response to filarial infection (Soboslay et al., 2006). CCL-17 knockout mice experienced a fourfold increase in microfilarial load, concomitant with an increase in tissue damage and vascular permeability from mast cell activation (Specht et al., 2011).

In *W. bancrofti*, new information regarding the possible immunomodulatory role of *Wolbachia* is coming to light. Shiny et al. observed that *Wolbachia* heat-shock protein-60 may contribute towards immune tolerance via the suppression of classical T-cell activation, in favour of T-reg cell activity (Shiny et al., 2011a). Furthermore, in microfilaraemic patients and those experiencing chronic pathologic manifestations, rWSP was reported to suppress lymphocyte proliferation and increase production of the T-reg-associated cytokines IL-10 and TGF-β, when compared to endemic normals (Shiny et al., 2011b). Since IL-10 modulates TLR-induced cytokine-production by human monocytes and dendritic cells (Hartog et al., 2011), perhaps *Wolbachia* may be capable of down-regulating the immune response that initially recognises it.

The down-regulation of classical T-cell responses by *Wolbachia* or its products is yet another example of the diverse immunomodulatory effects that it can exert on the invertebrate or mammalian host. However, it is unclear whether a Th-1-suppressing effect from *Wolbachia* is consistent with our hypothesis. Indeed, if this were the dominant effect evident in the *Onchocerca* nodule, any neutrophil stimulus from *Wolbachia* would presumably be mitigated. Thus, the differences in the way

Wolbachia exerts its immunomodulatory mechanisms may lie in the worm habitat, because the unique neutrophilic infiltration occurring so densely around sessile worms is only evident in the nodule-forming, *Wolbachia*-replete species (Brattig et al., 2001; Wildenburg et al., 1997). In contrast, in *O. flexuosa,* the only nodule-forming aposymbiotic filarial species known to date, the cellular composition adjacent to worms represents an example of a classical anti-helminthic Th-2 response, constituting many eosinophils (Wildenburg et al., 1997) and also giant cells (Brattig et al., 2001). Of further interest is that *O. flexuosa* once harboured *Wolbachia*, as evidenced by the finding of over 100 *Wolbachia*–like DNA fragments during the partial sequencing of the *O. flexuosa* genome (McNulty et al., 2010).

Onchocerca flexuosa is hypothesised to be a basal species within a relatively recent genus of filarial nematodes (Krueger et al., 2007). In contrast, ancestral lineages within the Filarioidea, such as *Setaria* and *Filaria* spp., are thought to have never harboured *Wolbachia* (Bain et al., 2008). It is also hypothesized that *Wolbachia* was acquired within the lineages leading to filariae harbouring the type C and D supergroups, which includes all nodule-forming *Onchocerca* spp., with the exception of *O. flexuosa* (Ferri et al., 2011). Interestingly, in the F supergroup (the only group to span arthropods and filariae), there is some evidence of secondary acquisition and recent capture of *Wolbachia* via lateral transfer (Ferri et al., 2011). Thus, the bear *Ursus thibetanus* can be co-infected with two species of filariae harbouring supergroup-F *Wolbachia*: *Mansonella akitensis* (a member of a genus in which *Wolbachia* infection is common), and *Cercopithifilaria japonica* (the only known *Cercopithifilaria* spp. infected with *Wolbachia*).

The apparent loss of *Wolbachia* over time by *O. flexuosa* suggests that "obligatory" symbioses within the Filarioidea may only represent the current status quo in some species. Furthermore, the recent discovery of the "patchy" distribution of *Wolbachia*, not only between species, but between individual worms within a species (*e.g.*, in *O. suzukii*), strengthens this hypothesis (Ferri et al., 2011). In light of this new evidence, a role as a defensive mutualist would possibly be a more appropriate description for *Wolbachia* of filariae. In the case of *O. flexuosa*, *Wolbachia* may have been lost before the establishment of any mutually dependent symbiosis (Ferri et al., 2011).

Thus, it seems that *Wolbachia* may co-evolve with its host by conferring relatively minor fitness advantages such as additional nutritional or immune evasion strategies, which are advantageous to the filarial host but not essential for filarial survival. Reciprocally, it would seem that the filarial host may either adopt these co-evolutionary advantages and eventually become dependent upon them, or perhaps instead develop (or retain) alternative methods of survival. The latter may have occurred with *O. flexuosa*, since its suggested lifespan (Plenge-Bonig et al., 1995) is similar to the time taken to reach sexual maturity in other nodule-forming filariae (Duke, 1993), and thus it may have adopted an accelerated maturation and reproductive cycle. Hence, while *O. ochengi* and *O. volvulus* survive for upwards of ten years within their mammalian hosts, *O. flexuosa* is purported to live for approximately one year (Plenge-Bonig et al., 1995).

The results of this study strengthen the hypothesis that, in the confines of the *O. ochengi* onchocercoma, *Wolbachia* manipulates the local host inflammatory response, which in a display of a defensive mutualism, serves to protect the worm host. Our findings strongly suggest that *Wolbachia* mediates protection of its filarial

host via recruitment of a neutrophilic infiltrate, which protects the worms from a classical effector cell response by eosinophils. This immune modulation may be specific to the *Onchocerca* nodule and the sessile female worms therein. However, because the bovine *O. ochengi* model represents the most closely related filarial analogue to the human disease, we hypothesise a similar mechanism for *O. volvulus*. This does indeed represent a novel variation of the defensive strategy by *Wolbachia*, adding to an impressive array of phenotypic characteristics that confer manipulative effects on a wide array of host species.

10.4. Indications for future studies

Despite the advancement of our genomic knowledge of the bovine (Elsik et al., 2009), still relatively little is known about key immunological mechanisms compared to murine model systems, and reagents may not be available. For example, in our cell migration study it was frustrating that bovine eotaxin was commercially unavailable, despite its efficacy as a positive control in a previous publication (Vogel et al., 2005).

Nevertheless, further cytokine profiling by qRT-PCR could include the analysis of ENA-78 and RANTES transcripts to attempt to elucidate their role in the treated and untreated nodule. Also of interest is bovine IL-10, which may be pertinent in the down-regulation of any Th-1 type response after *Wolbachia* depletion, although published data suggest that in the bovine, IL-10 does not selectively suppress Th-1 cells (Brown et al., 1994).

Cytokine protein assays of supernatants from bovine granulocyte and macrophage cultures following exposure to treated and untreated *O. ochengi* extracts could help to determine their role, and may assist in unveiling cytokine networks between these

cells. ELISA assays have been developed for bovine IL-10 (Kwong et al., 2002), and ELISA kits are available commercially for bovine IL-8, IL-5 (both from Gentaur) and eotaxin (Novatein Bio). However, the problems of reproducing the chronicity of *in vivo* events in an *in-vitro* system are unfortunately unavoidable, and must be taken into account. Alternatively, quantification of cytokines *ex-vivo* could utilise immunogold localisation of preformed cytokines ultrastructurally by TEM. Immunogold localisation has previously been used to locate TNF-α in human eosinophils (Beil et al., 1993) and IL-8 in mast cells (Grutzkau et al., 1997). An advantage of this approach is that the cytokines can be localised to specific granule locations; but disadvantages include the relative cost, and the limitation of detecting only stored, rather than released, cytokines.

Nodule sections could also be stained for the presence of TGF-β, since in tumours, TGF-β has been shown to be responsible for the upregulation of tumor-associated "N2" neutrophils, which are purported to produce lower levels of proinflammatory cytokines (Fridlender et al., 2009). A similar neutrophil phenotype may be evident in the *Onchocerca* nodule, as the neutrophils observed are not associated with high levels of activity such as necrosis and pus formation.

To advance our *in-vitro* cell migration assays, recombinant proteins could be tested in addition to whole worm homogenates. This would decipher the chemoattractive properties of specific filarial or *Wolbachia* components, and thus may provide a clearer picture of the sequence of events in the nodule. To identify any suppressive effects between granulocyte subsets, eosinophils could be pre-incubated with supernatants from neutrophils previously exposed to *O. ochengi* or *Wolbachia*. These

eosinophils could then be assayed within *in-vitro* chambers to assess their chemotactic response towards worm products, compared to unexposed cells.

To further our understanding of the effects of antibiotics on filariae and the influence of *Wolbachia*, it would be interesting to assess the production and release of E/S products in the face of antibiotic treatment and *Wolbachia* depletion. This would build upon the work that has already begun in this field, which determined that the immunomodulatory effects of filarial E/S products were abrogated via inhibition of their release by ivermectin treatment (Moreno et al., 2010).

Proteomic level analysis of *O. ochengi* is already underway at the University of Liverpool, and the data generated from this will be of enormous value in further elucidating some of the filarial interactions with both the bovine immune system and with *Wolbachia*. A whole transcriptome analysis of intact nodules before and after treatment would also provide key information on the interplay between *Wolbachia*, the worm and the bovine host under natural conditions and during chemotherapy.

11. List of references

Abasht, B., Kaiser, M.G., Lamont, S.J., 2008. Toll-like receptor gene expression in cecum and spleen of advanced intercross line chicks infected with *Salmonella enterica* serovar Enteritidis. Vet. Immunol. Immunopathol. **123**, 314-323.

Abraham, D., Leon, O., Schnyder-Candrian, S., Wang, C.C., Galioto, A.M., Kerepesi, L.A., Lee, J.J., Lustigman, S., 2004. Immunoglobulin E and eosinophil-dependent protective immunity to larval *Onchocerca* volvulus in mice immunized with irradiated larvae. Infect. Immun. **72**, 810-817.

Ackerman, S.J., Kephart, G.M., Francis, H., Awadzi, K., Gleich, G.J., Ottesen, E.A., 1990. Eosinophil degranulation. An immunologic determinant in the pathogenesis of the Mazzotti reaction in human onchocerciasis. J. Immunol. **144**, 3961-3969.

Ackerman, S.J., Liu, L., Kwatia, M.A., Savage, M.P., Leonidas, D.D., Swaminathan, G.J., Acharya, K.R., 2002. Charcot-Leyden crystal protein (galectin-10) is not a dual function galectin with lysophospholipase activity but binds a lysophospholipase inhibitor in a novel structural fashion. J. Biol. Chem. **277**, 14859-14868.

Aderem, A., Ulevitch, R.J., 2000. Toll-like receptors in the induction of the innate immune response. Nature **406**, 782-787.

Aebischer, C.P., Pasche, I., Jorg, A., 1993. Nanomolar arachidonic acid influences the respiratory burst in eosinophils and neutrophils induced by GTP-binding protein. A comparative study of the respiratory burst in bovine eosinophils and neutrophils. Eur. J. Biochem. **218**, 669-677.

Agosti, J.M., Altman, L.C., Ayars, G.H., Loegering, D.A., Gleich, G.J., Klebanoff, S.J., 1987. The injurious effect of eosinophil peroxidase, hydrogen peroxide, and halides on pneumocytes in vitro. J. Allergy Clin. Immunol. **79**, 496-504.

Ahmad-Nejad, P., Hacker, H., Rutz, M., Bauer, S., Vabulas, R.M., Wagner, H., 2002. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. Eur. J. Immunol. **32**, 1958-1968.

Ahuja, S.K., Lee, J.C., Murphy, P.M., 1996. CXC chemokines bind to unique sets of selectivity determinants that can function independently and are broadly distributed on multiple domains of human interleukin-8 receptor B. Determinants of high affinity binding and receptor activation are distinct. J. Biol. Chem. **271**, 225-232.

Akira, S., Takeda, K., Kaisho, T., 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat. Immunol. **2**, 675-680.

Al-Qaoud, K.M., Pearlman, E., Hartung, T., Klukowski, J., Fleischer, B., Hoerauf, A., 2000. A new mechanism for IL-5-dependent helminth control: neutrophil accumulation and neutrophil-mediated worm encapsulation in murine filariasis are abolished in the absence of IL-5. Int. Immunol. **12**, 899-908.

Albiez, E.J., Walter, G., Kaiser, A., Newland, H.S., White, A.T., Greene, B.M., Taylor, H.R., Buttner, D.W., 1988. Effects of high doses of diethylcarbamazine on adult *Onchocerca volvulus* examined by the collagenase technique and by histology. Trop. Med. Parasitol. **39**, 87-92.

Aliprantis, A.O., Yang, R.B., Mark, M.R., Suggett, S., Devaux, B., Radolf, J.D., Klimpel, G.R., Godowski, P., Zychlinsky, A., 1999. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science **285**, 736-739.

Allen, J.E., Adjei, O., Bain, O., Hoerauf, A., Hoffmann, W.H., Makepeace, B.L., Schulz-Key, H., Tanya, V.N., Trees, A.J., Wanji, S., Taylor, D.W., 2008. Of mice, cattle, and humans: the immunology and treatment of river blindness. PLoS. Negl. Trop. Dis. **2**, e217.

Allmann-Iselin, I., Car, B.D., Zwahlen, R.D., Muellerschupbach, R., Wyderwalther, M., Steckholzer, U., Walz, A., 1994. Bovine ENA, A New Monocyte-Macrophage Derived Cytokine of the Interleukin-8 Family - Structure, Function, and Expression in Acute Pulmonary Inflammation. American Journal of Pathology **145**, 1382-1389.

Anderson, K.V., 2000. Toll signaling pathways in the innate immune response. Curr. Opin. Immunol. **12**, 13-19.

Athens, J.W., Haab, O.P., Raab, S.O., Mauer, A.M., Ashenbrucker, H., Cartwright, G.E., Wintrobe, M.M., 1961. Leukokinetic studies. IV. The total blood, circulating and marginal granulocyte pools and the granulocyte turnover rate in normal subjects. J. Clin. Invest **40**, 989-995.

Atta el Mannan, A.M., Hussein, H.S., el, S.K., Magzoub, M., 1984. *Onchocerca armillata*: prevalence and pathology in Sudanese cattle. Ann. Trop. Med. Parasitol. **78**, 619-625.

Attout, T., Hoerauf, A., Denece, G., Debrah, A.Y., Marfo-Debrekyei, Y., Boussinesq, M., Wanji, S., Martinez, V., Mand, S., Adjei, O., Bain, O., Specht, S., Martin, C., 2009. Lymphatic vascularisation and involvement of Lyve-1+ macrophages in the human *Onchocerca* nodule. PLoS. One. **4**, e8234.

Aust, G., Brylla, E., Lehmann, I., Kiessling, S., Spanel-Borowski, K., 1999. Cloning of bovine RANTES mRNA and its expression and regulation in ovaries in the periovulatory period. FEBS Lett. **463**, 160-164.

Awadzi, K., 2003. Clinical picture and outcome of Serious Adverse Events in the treatment of Onchocerciasis. Filaria. J. **2** Suppl 1, S6.

Babu, S., Bhat, S.Q., Kumar, N.P., Lipira, A.B., Kumar, S., Karthik, C., Kumaraswami, V., Nutman, T.B., 2009. Filarial Lymphedema Is Characterized by Antigen-Specific Th1 and Th17 Proinflammatory Responses and a Lack of Regulatory T Cells. Plos Neglected Tropical Diseases **3**. 4, e420.

Baggiolini, M, 1972. Enzymes of Granules of Polymorphonuclear Leukocytes and Their Functions. Enzyme **13**, 1, 132-160.

Baggiolini, M., Dahinden, C.A., 1994. CC chemokines in allergic inflammation. Immunol. Today **15**, 127-133.

Baggiolini, M., Dewald, B., Moser, B., 1997. Human chemokines: an update. Annu. Rev. Immunol. **15**, 675-705.

Baggiolini, M., Walz, A., Kunkel, S.L., 1989. Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. J. Clin. Invest **84**, 1045-1049.

Bain, O., 1981. [The genus *Onchocherca* : hypothesis on its evolution and a key to the species (author's transl)]. Ann. Parasitol. Hum. Comp **56**, 503-526.

Bain, O., 2002. Evolutionary relationships among filarial nematodes. World Class Parasites: The Filaria Kluwer, U.S.A **5**, 21-30.

Bain, O., Casiraghi, M., Martin, C., Uni, S., 2008. The nematoda Filarioidea: critical analysis linking molecular and traditional approaches. Parasite **15**, 342-348.

Bainton, D.F., Farquhar, M.G., 1966. Origin of granules in polymorphonuclear leukocytes. Two types derived from opposite faces of the Golgi complex in developing granulocytes. J. Cell Biol. **28**, 277-301.

Balentien, E., Han, J.H., Thomas, H.G., Wen, D.Z., Samantha, A.K., Zachariae, C.O., Griffin, P.R., Brachmann, R., Wong, W.L., Matsushima, K., ., 1990. Recombinant expression, biochemical characterization, and biological activities of the human MGSA/gro protein. Biochemistry **29**, 10225-10233.

Balic, A., Cunningham, C.P., Meeusen, E.N., 2006. Eosinophil interactions with Haemonchus contortus larvae in the ovine gastrointestinal tract. Parasite Immunol. **28**, 107-115.

Bandi, C., Anderson, T.J., Genchi, C., Blaxter, M.L., 1998. Phylogeny of *Wolbachia* in filarial nematodes. Proc. Biol. Sci. **265**, 2407-2413.

Bandi, C., McCall, J.W., Genchi, C., Corona, S., Venco, L., Sacchi, L., 1999. Effects of tetracycline on the filarial worms *Brugia pahangi* and *Dirofilaria immitis* and their bacterial endosymbionts *Wolbachia*. Int. J. Parasitol. 29, 357-364.

Barber, M.R., Yang, T.J., 1998. Chemotactic activities in nonmastitic and mastitic mammary secretions: presence of interleukin-8 in mastitic but not nonmastitic secretions. Clin. Diagn. Lab Immunol. **5**, 82-86.

Barot-Ciorbaru, R., Brochier, J., Miyawaki, T., Preudhomme, J.L., Petit, J.F., Bona, C., Taniguchi, N., Revillard, J.P., 1985. Stimulation of Human Lymphocytes-B by *Nocardia*-Delipidated Cell Mitogen and Derived Fractions from N-Opaca - Structure-Activity Relationship. Journal of Immunology **135**, 3277-3283.

Barr, K.L., Hearne, L.B., Briesacher, S., Clark, T.L., Davis, G.E., 2010. Microbial symbionts in insects influence down-regulation of defense genes in maize. PLoS One **5**, e11339.

Basanez, M.G., Pion, S.D., Churcher, T.S., Breitling, L.P., Little, M.P., Boussinesq, M., 2006. River blindness: a success story under threat? PLoS. Med. **3**, e371.

Bazan, J.F., Bacon, K.B., Hardiman, G., Wang, W., Soo, K., Rossi, D., Greaves, D.R., Zlotnik, A., Schall, T.J., 1997. A new class of membrane-bound chemokine with a CX3C motif. Nature **385**, 640-644.

Bazzocchi, C., Ceciliani, F., McCall, J.W., Ricci, I., Genchi, C., Bandi, C., 2000. Antigenic role of the endosymbionts of filarial nematodes: IgG response against the *Wolbachia* surface protein in cats infected with *Dirofilaria immitis*. Proc. Biol. Sci. **267**, 2511-2516.

Bazzocchi, C., Genchi, C., Paltrinieri, S., Lecchi, C., Mortarino, M., Bandi, C., 2003. Immunological role of the endosymbionts of *Dirofilaria immitis*: the Wolbachia surface protein activates canine neutrophils with production of IL-8. Vet. Parasitol. **117**, 73-83.

Bazzocchi, C., Mortarino, M., Grandi, G., Kramer, L.H., Genchi, C., Bandi, C., Genchi, M., Sacchi, L., McCall, J.W., 2008. Combined ivermectin and doxycycline treatment has microfilaricidal and adulticidal activity against *Dirofilaria immitis* in experimentally infected dogs. Int. J. Parasitol. **38**, 1401-1410.

Bazzoni, F., Cassatella, M.A., Laudanna, C., Rossi, F., 1991a. Phagocytosis of opsonized yeast induces tumor necrosis factor-alpha mRNA accumulation and protein release by human polymorphonuclear leukocytes. J. Leukoc. Biol. **50**, 223-228.

Bazzoni, F., Cassatella, M.A., Rossi, F., Ceska, M., Dewald, B., Baggiolini, M., 1991b. Phagocytosing neutrophils produce and release high amounts of the neutrophil-activating peptide 1/interleukin 8. J. Exp. Med. **173**, 771-774.

Becker, S., Quay, J., Koren, H.S., Haskill, J.S., 1994. Constitutive and stimulated MCP-1, GRO alpha, beta, and gamma expression in human airway epithelium and bronchoalveolar macrophages. Am. J. Physiol **266**, L278-L286.

Beil, W.J., Weller, P.F., Tzizik, D.M., Galli, S.J., Dvorak, A.M., 1993. Ultrastructural Immunogold Localization of Tumor-Necrosis-Factor-Alpha to the Matrix Compartment of Eosinophil Secondary Granules in Patients with

Idiopathic Hypereosinophilic Syndrome. Journal of Histochemistry & Cytochemistry **41**, 1611-1615.

Belkaid, Y., Blank, R.B., Suffia, I., 2006. Natural regulatory T cells and parasites: a common quest for host homeostasis. Immunol. Rev. **212**, 287-300.

Bennett, J.L., Williams, J.F., Dave, V., 1988. Pharmacology of ivermectin. Parasitol. Today **4**, 226-228.

Bennuru, S., Semnani, R., Meng, Z., Ribeiro, J.M.C., Veenstra, T.D., Nutman, T.B., 2009. *Brugia malayi* Excreted/Secreted Proteins at the Host/Parasite Interface: Stage- and Gender-Specific Proteomic Profiling. Plos Neglected Tropical Diseases **3**, 4 e410.

Bhargava, K.K., Le, T.N., Cerami, A., Eaton, J.W., 1983. Effect of arsenical drugs on glutathione metabolism of *Litomosoides carinii*. Mol. Biochem. Parasitol. **9**, 29-35.

Bleiss, W., Oberlander, U., Hartmann, S., Adam, R., Marko, A., Schonemeyer, A., Lucius, R., 2002. Protective immunity induced by irradiated third-stage larvae of the filaria *Acanthocheilonema viteae* is directed against challenge third-stage larvae before molting. J. Parasitol. **88**, 264-270.

Bochner, B.S., Klunk, D.A., Sterbinsky, S.A., Coffman, R.L., Schleimer, R.P., 1995. IL-13 selectively induces vascular cell adhesion molecule-1 expression in human endothelial cells. J. Immunol. **154**, 799-803.

Bosshardt, S.C., McCall, J.W., Coleman, S.U., Jones, K.L., Petit, T.A., Klei, T.R., 1993. Prophylactic activity of tetracycline against *Brugia pahangi* infection in jirds (Meriones unguiculatus). J. Parasitol. **79**, 775-777.

Bougarn, S., Cunha, P., Harmache, A., Fromageau, A., Gilbert, F.B., Rainard, P., 2010. Muramyl dipeptide synergizes with *Staphylococcus aureus* lipoteichoic acid to recruit neutrophils in the mammary gland and to stimulate mammary epithelial cells. Clin. Vaccine Immunol. **17**, 1797-1809.

Bourtzis, K., Nirgianaki, A., Markakis, G., Savakis, C., 1996. *Wolbachia* infection and cytoplasmic incompatibility in *Drosophila* species. Genetics **144**, 1063-1073.

Boussinesq, M., 2006. Loiasis. Ann. Trop. Med. Parasitol. **100**, 715-731.

Brattig, N.W., 2004a. Pathogenesis and host responses in human onchocerciasis: impact of *Onchocerca* filariae and *Wolbachia* endobacteria. Microbes. Infect. **6**, 113-128.

Brattig, N.W., Abakar, A.Z., Geisinger, F., Kruppa, T.F., 1995. Cell-adhesion molecules expressed by activated eosinophils in *Onchocerca volvulus* infection. Parasitol. Res. **81**, 398-402.

Brattig, N.W., Bazzocchi, C., Kirschning, C.J., Reiling, N., Buttner, D.W., Ceciliani, F., Geisinger, F., Hochrein, H., Ernst, M., Wagner, H., Bandi, C., Hoerauf,

A., 2004b. The major surface protein of *Wolbachia* endosymbionts in filarial nematodes elicits immune responses through TLR2 and TLR4. J. Immunol. **173**, 437-445.

Brattig, N.W., Buttner, D.W., Hoerauf, A., 2001. Neutrophil accumulation around *Onchocerca* worms and chemotaxis of neutrophils are dependent on *Wolbachia* endobacteria. Microbes. Infect. **3**, 439-446.

Brattig, N.W., Tischendorf, F.W., Strote, G., Medina-De la Garza CE, 1991. Eosinophil-larval-interaction in onchocerciasis: heterogeneity of in vitro adherence of eosinophils to infective third and fourth stage larvae and microfilariae of *Onchocerca volvulus*. Parasite Immunol. **13**, 13-22.

Bronsvoort, B.M., Renz, A., Tchakoute, V., Tanya, V.N., Ekale, D., Trees, A.J., 2005. Repeated high doses of avermectins cause prolonged sterilisation, but do not kill, *Onchocerca ochengi* adult worms in African cattle. Filaria. J. **4**, 8.

Bronsvoort, B.M., Makepeace, B.L., Renz, A., Tanya, V.N., Fleckenstein, L., Ekale, D., Trees, A.J., 2008. UMF-078: A modified flubendazole with potent macrofilaricidal activity against *Onchocerca ochengi* in African cattle. Parasit. Vectors. **1**, 18.

Brown, G.B., Roth, J.A., 1991. Comparison of the response of bovine and human neutrophils to various stimuli. Vet. Immunol. Immunopathol. **28**, 201-218.

Brown, W.C., Woods, V.M., Chitko-McKown, C.G., Hash, S.M., Rice-Ficht, A.C., 1994. Interleukin-10 is expressed by bovine type 1 helper, type 2 helper, and unrestricted parasite-specific T-cell clones and inhibits proliferation of all three subsets in an accessory-cell-dependent manner. Infect. Immun. **62**, 4697-4708.

Brydon, L.J., Gooday, G.W., Chappell, L.H., King, T.P., 1987. Chitin in egg shells of *Onchocerca gibsoni* and *Onchocerca volvulus*. Mol. Biochem. Parasitol. **25**, 267-272.

Burke, J.M., Ganley-Leal, L.M., Khatri, A., Wetzler, L.M., 2007. *Neisseria meningitidis* PorB, a TLR2 ligand, induces an antigen-specific eosinophil recall response: potential adjuvant for helminth vaccines? J. Immunol. **179**, 3222-3230.

Burke-Gaffney, A., Hellewell, P.G., 1996. Eotaxin stimulates eosinophil adhesion to human lung microvascular endothelial cells. Biochem. Biophys. Res. Commun. **227**, 35-40.

Burns, A.R., Bowden, R.A., Abe, Y., Walker, D.C., Simon, S.I., Entman, M.L., Smith, C.W., 1999. P-selectin mediates neutrophil adhesion to endothelial cell borders. J. Leukoc. Biol. **65**, 299-306.

Butcher, E.C., 1991. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. Cell **67**, 1033-1036.

Butterworth, A.E., Sturrock, R.F., Houba, V., Mahmoud, A.A.F., Sher, A., Rees, P.H., 1975. Eosinophils As Mediators of Antibody-Dependent Damage to *Schistosomula*. Nature **256**, 727-729.

Buttner, D.W., Racz, P., 1983. Macro- and microfilariae in nodules from onchocerciasis patients in the Yemen Arab Republic. Tropenmed. Parasitol. **34**, 113-121.

Buttner, D.W., Wanji, S., Bazzocchi, C., Bain, O., Fischer, P., 2003. Obligatory symbiotic *Wolbachia* endobacteria are absent from *Loa loa*. Filaria. J. **2**, 10.

Carlow, C.K., Dobinson, A.R., Bianco, A.E., 1988. Parasite-specific immune responses to *Onchocerca lienalis* microfilariae in normal and immunodeficient mice. Parasite Immunol. **10**, 309-322.

Casiraghi, M., Bain, O., Guerrero, R., Martin, C., Pocacqua, V., Gardner, S.L., Franceschi, A., Bandi, C., 2004. Mapping the presence of *Wolbachia pipientis* on the phylogeny of filarial nematodes: evidence for symbiont loss during evolution. Int. J. Parasitol. **34**, 191-203.

Caswell, J.L., Middleton, D.M., Gordon, J.R., 1999. Production and functional characterization of recombinant bovine interleukin-8 as a specific neutrophil activator and chemoattractant. Vet. Immunol. Immunopathol. **67**, 327-340.

Caswell, J.L., Middleton, D.M., Gordon, J.R., 2001. The importance of interleukin-8 as a neutrophil chemoattractant in the lungs of cattle with pneumonic pasteurellosis. Canadian Journal of Veterinary Research-Revue Canadienne de Recherche Veterinaire **65**, 229-232.

Chambers, W.H., Taylor, J.R., Klesius, P.H., 1983. Isolation of bovine polymorphonuclear leukocytes by density gradient centrifugation. Vet. Immunol. Immunopathol. **5**, 197-202.

Chambers, W.H., Washburn, S.M., Campos, M., Klesius, P.H., 1985. Isolation of bovine eosinophils by density gradient centrifugation. Am. J. Vet. Res. **46**, 154-156.

Chandrashekar, R., Rao, U.R., Subrahmanyam, D., 1985. Serum dependent cell-mediated immune reactions to *Brugia pahangi* infective larvae. Parasite Immunol. **7**, 633-641.

Cherry, W.B., Yoon, J., Bartemes, K.R., Iijima, K., Kita, H., 2008. A novel IL-1 family cytokine, IL-33, potently activates human eosinophils. J. Allergy Clin. Immunol. **121**, 1484-1490.

Chiers, K., Weyens, P., Deprez, P., Van, H.M., Meulemans, G., Baert, K., Croubels, S., De, B.P., Ducatelle, R., 2004. Lingual and pharyngeal paralysis due to acute doxycycline intoxication in veal calves. Vet. Rec. **155**, 25-26.

Chijioke, C.P., Umeh, R.E., Mbah, A.U., Nwonu, P., Fleckenstein, L.L., Okonkwo, P.O., 1998. Clinical pharmacokinetics of suramin in patients with onchocerciasis. Eur. J. Clin. Pharmacol. **54**, 249-251.

Chopra, I., Roberts, M., 2001. Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiology and Molecular Biology Reviews **65**, 2, 232-260.

Coeffier, E., Joseph, D., Vargaftig, B.B., 1991. Activation of guinea pig eosinophils by human recombinant IL-5. Selective priming to platelet-activating factor-acether and interference of its antagonists. J. Immunol. **147**, 2595-2602.

Collins, P.D., Marleau, S., Griffiths-Johnson, D.A., Jose, P.J., Williams, T.J., 1995. Cooperation between interleukin-5 and the chemokine eotaxin to induce eosinophil accumulation in vivo. J. Exp. Med. **182**, 1169-1174.

Cook, R.M., Musgrove, N.R., Smith, H., 1988. Relationship between neutrophil infiltration and tissue eosinophilia in the rat. Int. Arch. Allergy Appl. Immunol. **87**, 105-108.

Cooper, P.J., Beck, L.A., Espinel, I., Deyampert, N.M., Hartnell, A., Jose, P.J., Paredes, W., Guderian, R.H., Nutman, T.B., 2000. Eotaxin and RANTES expression by the dermal endothelium is associated with eosinophil infiltration after ivermectin treatment of onchocerciasis. Clin. Immunol. **95**, 51-61.

Copeman, D.B., 1979. Evaluation of the Bovine - *Onchocerca-Gibsoni*, *Onchocerca-Gutturosa* Model As A Tertiary Screen for Drugs Against *Onchocerca-Volvulus* in Man. Tropenmedizin und Parasitologie **30**, 469-474.

Cross, H.F., Bronsvoort, B.M., Wahl, G., Renz, A., Achu-Kwi, D., Trees, A.J., 1997. The entry of ivermectin and suramin into *Onchocerca ochengi* nodules. Ann. Trop. Med. Parasitol. **91**, 393-401.

Cross, H.F., Haarbrink, M., Egerton, G., Yazdanbakhsh, M., Taylor, M.J., 2001. Severe reactions to filarial chemotherapy and release of *Wolbachia* endosymbionts into blood. Lancet **358**, 1873-1875.

Da Silva, C.A., Hartl, D., Liu, W., Lee, C.G., Elias, J.A., 2008. TLR-2 and IL-17A in chitin-induced macrophage activation and acute inflammation. J. Immunol. **181**, 4279-4286.

Dangi, A., Vedi, S., Nag, J.K., Paithankar, S., Singh, M.P., Kar, S.K., Dube, A., Misra-Bhattacharya, S., 2009. Tetracycline treatment targeting *Wolbachia* affects expression of an array of proteins in *Brugia malayi* parasite. Proteomics **9**, 4192-4208.

Debrah, A.Y., Mand, S., Marfo-Debrekyei, Y., Batsa, L., Pfarr, K., Buttner, M., Adjei, O., Buttner, D., Hoerauf, A., 2007. Macrofilaricidal effect of 4 weeks of treatment with doxycycline on *Wuchereria bancrofti*. Tropical Medicine & International Health **12**, 1433-1441.

Debrah, A.Y., Mand, S., Specht, S., Marfo-Debrekyei, Y., Batsa, L., Pfarr, K., Larbi, J., Lawson, B., Taylor, M., Adjei, O., Hoerauf, A., 2006. Doxycycline

reduces plasma VEGF-C/sVEGFR-3 and improves pathology in lymphatic filariasis. PLoS. Pathog. **2**, e92.

Dedeine, F., Bouletreau, M., Vavre, F., 2005. *Wolbachia* requirement for oogenesis: occurrence within the genus *Asobara* (Hymenoptera, Braconidae) and evidence for intraspecific variation in *A. tabida*. Heredity **95**, 394-400.

Denise, H., Giroud, C., Barrett, M.P., Baltz, T., 1999. Affinity chromatography using trypanocidal arsenical drugs identifies a specific interaction between glycerol-3-phosphate dehydrogenase from *Trypanosoma brucei* and Cymelarsan. Eur. J. Biochem. **259**, 339-346.

Devaney, E., Gillan, V., Wheatley, I., Jenson, J., O'Connor, R., Balmer, P., 2002. Interleukin-4 influences the production of microfilariae in a mouse model of *Brugia* infection. Parasite Immunol. **24**, 29-37.

Diacovo, T.G., Roth, S.J., Buccola, J.M., Bainton, D.F., Springer, T.A., 1996. Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18. Blood **88**, 146-157.

Diaz, A., Allen, J.E., 2007. Mapping immune response profiles: the emerging scenario from helminth immunology. Eur. J. Immunol. **37**, 3319-3326.

Dickerson, C.W., Thompson, P.E., 1966. Effects of Mel W against *Litomosoides carinii* in Mongolian gerbils. J. Parasitol. **52**, 1217-1218.

Doetze, A., Satoguina, J., Burchard, G., Rau, T., Loliger, C., Fleischer, B., Hoerauf, A., 2000. Antigen-specific cellular hyporesponsiveness in a chronic human helminth infection is mediated by T(h)3/T(r)1-type cytokines IL-10 and transforming growth factor-beta but not by a T(h)1 to T(h)2 shift. Int. Immunol. **12**, 623-630.

Donnelly, J.J., Rockey, J.H., Taylor, H.R., Soulsby, E.J., 1985. Onchocerciasis: experimental models of ocular disease. Rev. Infect. Dis. 7, 820-825.

Donofrio, G., Capocefalo, A., Franceschi, V., Price, S., Cavirani, S., Sheldon, I.M., 2010. The Chemokine IL8 Is Up-Regulated in Bovine Endometrial Stromal Cells by the BoHV-4 IE2 Gene Product, ORF50/Rta: A Step Ahead Toward a Mechanism for BoHV-4 Induced Endometritis. Biology of Reproduction **83**, 919-928.

Dor, P.J., Ackerman, S.J., Gleich, G.J., 1984. Charcot-Leyden crystal protein and eosinophil granule major basic protein in sputum of patients with respiratory diseases. Am. Rev. Respir. Dis. **130**, 1072-1077.

Dubucquoi, S., Desreumaux, P., Janin, A., Klein, O., Goldman, M., Tavernier, J., Capron, M., Capron, A., 1994. Interleukin-5 Synthesis by Eosinophils - Association with Granules and Immunoglobulin-Dependent Secretion. Journal of Experimental Medicine **179**, 703-708.

Duffus, W.P., Thorne, K., Oliver, R., 1980. Killing of juvenile *Fasciola hepatica* by purified bovine eosinophil proteins. Clin. Exp. Immunol. **40**, 336-344.

Duke, B.O., 1968. The effects of drugs on *Onchocerca volvulus*. 1. Methods of assessment, population dynamics of the parasite and the effects of diethylcarbamazine. Bull. World Health Organ **39**, 137-146.

Duke, B.O., 1993. The population dynamics of *Onchocerca volvulus* in the human host. Trop. Med. Parasitol. **44**, 61-68.

Eagle, H., 1939. The effect of sulfhydryl compounds on the antispirochetal action of arsenic, bismuth, and mercury compounds in vitro. Journal of Pharmacology and Experimental Therapeutics **66**, 436-448.

Egyed, Z., Sreter, T., Szell, Z., Nyiro, G., Dobos-Kovacs, M., Marialigeti, K., Varga, I., 2002. Electron microscopic and molecular identification of Wolbachia endosymbionts from *Onchocerca lupi*: implications for therapy. Vet. Parasitol. **106**, 75-82.

Elsik, C.G., Tellam, R.L., Worley, K.C., Gibbs, R.A., Muzny, D.M., Weinstock, G.M., Adelson, D.L., Eichler, E.E., Elnitski, L., Guigo, R., Hamernik, D.L., Kappes, S.M., Lewin, H.A., Lynn, D.J., Nicholas, F.W., Reymond, A., Rijnkels, M., Skow, L.C., Zdobnov, E.M., Schook, L., Womack, J., Alioto, T., Antonarakis, S.E., Astashyn, A., Chapple, C.E., Chen, H.C., Chrast, J., Camara, F., Ermolaeva, O., Henrichsen, C.N., Hlavina, W., Kapustin, Y., Kiryutin, B., Kitts, P., Kokocinski, F., Landrum, M., Maglott, D., Pruitt, K., Sapojnikov, V., Searle, S.M., Solovyev, V., Souvorov, A., Ucla, C., Wyss, C., Anzola, J.M., Gerlach, D., Elhaik, E., Graur, D., Reese, J.T., Edgar, R.C., McEwan, J.C., Payne, G.M., Raison, J.M., Junier, T., Kriventseva, E.V., Eyras, E., Plass, M., Donthu, R., Larkin, D.M., Reecy, J., Yang, M.Q., Chen, L., Cheng, Z., Chitko-McKown, C.G., Liu, G.E., Matukumalli, L.K., Song, J., Zhu, B., Bradley, D.G., Brinkman, F.S., Lau, L.P., Whiteside, M.D., Walker, A., Wheeler, T.T., Casey, T., German, J.B., Lemay, D.G., Maqbool, N.J., Molenaar, A.J., Seo, S., Stothard, P., Baldwin, C.L., Baxter, R., Brinkmeyer-Langford, C.L., Brown, W.C., Childers, C.P., Connelley, T., Ellis, S.A., Fritz, K., Glass, E.J., Herzig, C.T., Iivanainen, A., Lahmers, K.K., Bennett, A.K., Dickens, C.M., Gilbert, J.G., Hagen, D.E., Salih, H., Aerts, J., Caetano, A.R., Dalrymple, B., Garcia, J.F., Gill, C.A., Hiendleder, S.G., Memili, E., Spurlock, D., Williams, J.L., Alexander, L., Brownstein, M.J., Guan, L., Holt, R.A., Jones, S.J., Marra, M.A., Moore, R., Moore, S.S., Roberts, A., Taniguchi, M., Waterman, R.C., Chacko, J., Chandrabose, M.M., Cree, A., Dao, M.D., Dinh, H.H., Gabisi, R.A., Hines, S., Hume, J., Jhangiani, S.N., Joshi, V., Kovar, C.L., Lewis, L.R., Liu, Y.S., Lopez, J., Morgan, M.B., Nguyen, N.B., Okwuonu, G.O., Ruiz, S.J., Santibanez, J., Wright, R.A., Buhay, C., Ding, Y., Dugan-Rocha, S., Herdandez, J., Holder, M., Sabo, A., Egan, A., Goodell, J., Wilczek-Boney, K., Fowler, G.R., Hitchens, M.E., Lozado, R.J., Moen, C., Steffen, D., Warren, J.T., Zhang, J., Chiu, R., Schein, J.E., Durbin, K.J., Havlak, P., Jiang, H., Liu, Y., Qin, X., Ren, Y., Shen, Y., Song, H., Bell, S.N., Davis, C., Johnson, A.J., Lee, S., Nazareth, L.V., Patel, B.M., Pu, L.L., Vattathil, S., Williams, R.L., Jr., Curry, S., Hamilton, C., Sodergren, E., Wheeler, D.A., Barris, W., Bennett, G.L.,

Eggen, A., Green, R.D., Harhay, G.P., Hobbs, M., Jann, O., Keele, J.W., Kent, M.P., Lien, S., McKay, S.D., McWilliam, S., Ratnakumar, A., Schnabel, R.D., Smith, T., Snelling, W.M., Sonstegard, T.S., Stone, R.T., Sugimoto, Y., Takasuga, A., Taylor, J.F., Van Tassell, C.P., Macneil, M.D., Abatepaulo, A.R., Abbey, C.A., Ahola, V., Almeida, I.G., Amadio, A.F., Anatriello, E., Bahadue, S.M., Biase, F.H., Boldt, C.R., Carroll, J.A., Carvalho, W.A., Cervelatti, E.P., Chacko, E., Chapin, J.E., Cheng, Y., Choi, J., Colley, A.J., de Campos, T.A., De, D.M., Santos, I.K., de Oliveira, C.J., Deobald, H., Devinoy, E., Donohue, K.E., Dove, P., Eberlein, A., Fitzsimmons, C.J., Franzin, A.M., Garcia, G.R., Genini, S., Gladney, C.J., Grant, J.R., Greaser, M.L., Green, J.A., Hadsell, D.L., Hakimov, H.A., Halgren, R., Harrow, J.L., Hart, E.A., Hastings, N., Hernandez, M., Hu, Z.L., Ingham, A., Iso-Touru, T., Jamis, C., Jensen, K., Kapetis, D., Kerr, T., Khalil, S.S., Khatib, H., Kolbehdari, D., Kumar, C.G., Kumar, D., Leach, R., Lee, J.C., 2009. The genome sequence of taurine cattle: a window to ruminant biology and evolution. Science **324**, 522-528.

Elsner, J., Hochstetter, R., Kimmig, D., Kapp, A., 1996. Human eotaxin represents a potent activator of the respiratory burst of human eosinophils. Eur. J. Immunol. **26**, 1919-1925.

Erger, R.A., Casale, T.B., 1995. Interleukin-8 is a potent mediator of eosinophil chemotaxis through endothelium and epithelium. Am. J. Physiol **268**, L117-L122.

Evans, T.G., 1995. Socioeconomic consequences of blinding onchocerciasis in west Africa. Bull. World Health Organ **73**, 495-506.

Fenollar, F., Maurin, M., Raoult, D., 2003. *Wolbachia pipientis* growth kinetics and susceptibilities to 13 antibiotics determined by immunofluorescence staining and real-time PCR. Antimicrob. Agents Chemother. **47**, 1665-1671.

Fernandez, H.N., Henson, P.M., Otani, A., Hugli, T.E., 1978. Chemotactic response to human C3a and C5a anaphylatoxins. I. Evaluation of C3a and C5a leukotaxis *in vitro* and under stimulated *in vivo* conditions. J. Immunol. **120**, 109-115.

Ferri, E., Bain, O., Barbuto, M., Martin, C., Lo, N., Uni, S., Landmann, F., Baccei, S.G., Guerrero, R., de Souza, L.S., Bandi, C., Wanji, S., Diagne, M., Casiraghi, M., 2011a. New insights into the evolution of *Wolbachia* infections in filarial nematodes inferred from a large range of screened species. PLoS. One. **6**, e20843.

Fischer, N., Czarnetzki, B.M., 1982. Comparative studies on eosinophil chemotactic factors during leukocyte migration under agarose. J. Invest Dermatol. **79**, 222-226.

Folkard, S.G., Bianco, A.E., 1995. Roles for both CD4+ and CD8+ T cells in protective immunity against *Onchocerca lienalis* microfilariae in the mouse. Parasite Immunol. **17**, 541-553.

Folkard, S.G., Hogarth, P.J., Taylor, M.J., Bianco, A.E., 1996. Eosinophils are the major effector cells of immunity to microfilariae in a mouse model of onchocerciasis. Parasitology **112** (Pt 3), 323-329.

Ford-Hutchinson, A.W., Bray, M.A., Doig, M.V., Shipley, M.E., Smith, M.J., 1980. Leukotriene B, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. Nature **286**, 264-265.

Foreman, K.E., Vaporciyan, A.A., Bonish, B.K., Jones, M.L., Johnson, K.J., Glovsky, M.M., Eddy, S.M., Ward, P.A., 1994. C5a-induced expression of P-selectin in endothelial cells. J. Clin. Invest **94**, 1147-1155.

Forsell, J.H., Kateley, J.R., Smith, C.W., 1985. Bovine neutrophils treated with chemotactic agents: morphologic changes. Am. J. Vet. Res. **46**, 1971-1974.

Foster, J., Ganatra, M., Kamal, I., Ware, J., Makarova, K., Ivanova, N., Bhattacharyya, A., Kapatral, V., Kumar, S., Posfai, J., Vincze, T., Ingram, J., Moran, L., Lapidus, A., Omelchenko, M., Kyrpides, N., Ghedin, E., Wang, S., Goltsman, E., Joukov, V., Ostrovskaya, O., Tsukerman, K., Mazur, M., Comb, D., Koonin, E., Slatko, B., 2005. The *Wolbachia* genome of *Brugia malayi*: Endosymbiont evolution within a human pathogenic nematode. Plos Biology **3**, 599-614.

Fouser, L.A., Wright, J.F., Dunussi-Joannopoulos, K., Collins, M., 2008. Th17 cytokines and their emerging roles in inflammation and autoimmunity. Immunol. Rev. **226**, 87-102.

Francis, H., Awadzi, K., Ottesen, E.A., 1985. The Mazzotti reaction following treatment of onchocerciasis with diethylcarbamazine: clinical severity as a function of infection intensity. Am. J. Trop. Med. Hyg. **34**, 529-536.

Franz, M., Schulz-Key, H., Copeman, D.B., 1987. Electron-microscopic observations on the female worms of six *Onchocerca* species from cattle and red deer. Parasitol. Res. **74**, 73-83.

Freeman, A.R., Lynn, D.J., Murray, C., Bradley, D.G., 2008. Detecting the effects of selection at the population level in six bovine immune genes. Bmc Genetics **6**, 9-62

Freiburghaus, J., Jorg, A., 1990. Isolation of bovine eosinophils and characterization of their leukotriene formation. Agents Actions **31**, 16-22.

Fridlender, Z.G., Sun, J., Kim, S., Kapoor, V., Cheng, G.J., Ling, L.N., Worthen, G.S., Albelda, S.M., 2009. Polarization of Tumor-Associated Neutrophil Phenotype by TGF-beta: "N1" versus "N2" TAN. Cancer Cell **16**, 183-194.

Fujiwara, K., Matsukawa, A., Ohkawara, S., Takagi, K., Yoshinaga, M., 2002. Functional distinction between CXC chemokines, interleukin-8 (IL-8), and growth related oncogene (GRO) alpha in neutrophil infiltration. Lab Invest **82**, 15-23.

Fukuda, T., Fukushima, Y., Numao, T., Ando, N., Arima, M., Nakajima, H., Sagara, H., Adachi, T., Motojima, S., Makino, S., 1996. Role of interleukin-4 and vascular cell adhesion molecule-1 in selective eosinophil migration into the airways in allergic asthma. Am. J. Respir. Cell Mol. Biol. **14**, 84-94.

Gale, L.M., McColl, S.R., 1999. Chemokines: extracellular messengers for all occasions? Bioessays **21**, 17-28.

Garcia-Zepeda, E.A., Rothenberg, M.E., Ownbey, R.T., Celestin, J., Leder, P., Luster, A.D., 1996. Human eotaxin is a specific chemoattractant for eosinophil cells and provides a new mechanism to explain tissue eosinophilia. Nat. Med. **2**, 449-456.

Gardon, J., Gardon-Wendel, N., Demanga, N., Kamgno, J., Chippaux, J.P., Boussinesq, M., 1997. Serious reactions after mass treatment of onchocerciasis with ivermectin in an area endemic for *Loa loa* infection. Lancet **350**, 18-22.

Genchi, C., Rinaldi, L., Cascone, C., Mortarino, M., Cringoli, G., 2005. Is heartworm disease really spreading in Europe? Vet. Parasitol. **133**, 137-148.

Gennaro, R., Dewald, B., Horisberger, U., Gubler, H.U., Baggiolini, M., 1983. A novel type of cytoplasmic granule in bovine neutrophils. J. Cell Biol. **96**, 1651-1661.

Ghedin, E., Hailemariam, T., DePasse, J.V., Zhang, X., Oksov, Y., Unnasch, T.R., Lustigman, S., 2009. Brugia malayi Gene Expression in Response to the Targeting of the *Wolbachia* Endosymbiont by Tetracycline Treatment. Plos Neglected Tropical Diseases **3**.

Ghedin, E., Wang, S., Spiro, D., Caler, E., Zhao, Q., Crabtree, J., Allen, J.E., Delcher, A.L., Guiliano, D.B., Miranda-Saavedra, D., Angiuoli, S.V., Creasy, T., Amedeo, P., Haas, B., El-Sayed, N.M., Wortman, J.R., Feldblyum, T., Tallon, L., Schatz, M., Shumway, M., Koo, H., Salzberg, S.L., Schobel, S., Pertea, M., Pop, M., White, O., Barton, G.J., Carlow, C.K., Crawford, M.J., Daub, J., Dimmic, M.W., Estes, C.F., Foster, J.M., Ganatra, M., Gregory, W.F., Johnson, N.M., Jin, J., Komuniecki, R., Korf, I., Kumar, S., Laney, S., Li, B.W., Li, W., Lindblom, T.H., Lustigman, S., Ma, D., Maina, C.V., Martin, D.M., McCarter, J.P., McReynolds, L., Mitreva, M., Nutman, T.B., Parkinson, J., Peregrin-Alvarez, J.M., Poole, C., Ren, Q., Saunders, L., Sluder, A.E., Smith, K., Stanke, M., Unnasch, T.R., Ware, J., Wei, A.D., Weil, G., Williams, D.J., Zhang, Y., Williams, S.A., Fraser-Liggett, C., Slatko, B., Blaxter, M.L., Scott, A.L., 2007. Draft genome of the filarial nematode parasite *Brugia malayi*. Science **317**, 1756-1760.

Gilbert, J., Nfon, C.K., Makepeace, B.L., Njongmeta, L.M., Hastings, I.M., Pfarr, K.M., Renz, A., Tanya, V.N., Trees, A.J., 2005. Antibiotic chemotherapy of onchocerciasis: in a bovine model, killing of adult parasites requires a sustained depletion of endosymbiotic bacteria (*Wolbachia* species). J. Infect. Dis. **192**, 1483-1493.

Gillan, V., Devaney, E., 2005. Regulatory T cells modulate Th2 responses induced by *Brugia pahangi* third-stage larvae. Infect. Immun. **73**, 4034-4042.

Gillette-Ferguson, I., Daehnel, K., Hise, A.G., Sun, Y., Carlson, E., Diaconu, E., McGarry, H.F., Taylor, M.J., Pearlman, E., 2007. Toll-like receptor 2 regulates CXC chemokine production and neutrophil recruitment to the cornea in *Onchocerca volvulus/Wolbachia*-induced keratitis. Infect. Immun. **75**, 5908-5915.

Gillette-Ferguson, I., Hise, A.G., McGarry, H.F., Turner, J., Esposito, A., Sun, Y., Diaconu, E., Taylor, M.J., Pearlman, E., 2004. *Wolbachia*-induced neutrophil activation in a mouse model of ocular onchocerciasis (river blindness). Infect. Immun. **72**, 5687-5692.

Glaser, R.L., Meola, M.A., 2010. The native *Wolbachia* endosymbionts of *Drosophila melanogaster* and *Culex quinquefasciatus* increase host resistance to West Nile virus infection. PLoS One **5**, e11977.

Glauert, A.M., Lammas, D.A., Duffus, W.P., 1985. Ultrastructural observations on the interaction in vitro between bovine eosinophils and juvenile *Fasciola hepatica*. Parasitology **91** (Pt 3), 459-470.

Gleich, G.J., Loegering, D.A., 1984. Immunobiology of eosinophils. Annu. Rev. Immunol. **2**, 429-459.

Godaly, G., Bergsten, G., Hang, L., Fischer, H., Frendeus, B., Lundstedt, A.C., Samuelsson, M., Samuelsson, P., Svanborg, C., 2001. Neutrophil recruitment, chemokine receptors, and resistance to mucosal infection. J. Leukoc. Biol. **69**, 899-906.

Goldammer, T., Zerbe, H., Molenaar, A., Schuberth, H.J., Brunner, R.M., Kata, S.R., Seyfert, H.M., 2004. Mastitis increases mammary mRNA abundance of beta-defensin 5, toll-like-receptor 2 (TLR2), and TLR4 but not TLR9 in cattle. Clin. Diagn. Lab Immunol. **11**, 174-185.

Gooday, G.W., Brydon, L.J., Chappell, L.H., 1988. Chitinase in Female *Onchocerca-Gibsoni* and Its Inhibition by Allosamidin. Molecular and Biochemical Parasitology **29**, 223-225.

Goodridge, H.S., Wilson, E.H., Harnett, W., Campbell, C.C., Harnett, M.M., Liew, F.Y., 2001. Modulation of macrophage cytokine production by ES-62, a secreted product of the filarial nematode *Acanthocheilonema viteae*. J. Immunol. **167**, 940-945.

Goto, F., Nakamura, S., Goto, K., Yoshinaga, M., 1984. Production of a lymphocyte proliferation potentiating factor by purified polymorphonuclear leucocytes from mice and rabbits. Immunology **53**, 683-692.

Graham, S.P., Trees, A.J., Collins, R.A., Moore, D.M., Guy, F.M., Taylor, M.J., Bianco, A.E., 2001. Down-regulated lymphoproliferation coincides with parasite maturation and with the collapse of both gamma interferon and

interleukin-4 responses in a bovine model of onchocerciasis. Infect. Immun. **69**, 4313-4319.

Greene, B.M., Taylor, H.R., Aikawa, M., 1981. Cellular killing of microfilariae of *Onchocerca volvulus*: eosinophil and neutrophil-mediated immune serum-dependent destruction. J. Immunol. **127**, 1611-1618.

Grewal, A.S., Babiuk, L.A., 1979. Induction, Isolation and Surface Marker Studies on Bovine Eosinophils. Journal of Immunological Methods **25**, 65-75.

Grobusch, M.P., Kombila, M., Autenrieth, I., Mehlhorn, H., Kremsner, P.G., 2003. No evidence of *Wolbachia* endosymbiosis with *Loa loa* and *Mansonella perstans*. Parasitol. Res. **90**, 405-408.

Grutzkau, A., KrugerKrasagakes, S., Kogel, H., Moller, A., Lippert, U., Henz, B.M., 1997. Detection of intracellular interleukin-8 in human mast cells: Flow cytometry as a guide for immunoelectron microscopy. Journal of Histochemistry & Cytochemistry **45**, 935-945.

Gu, Y., Hu, X.J., Liu, C.F., Qv, X., Xu, C.G., 2008. Interleukin (IL)-17 promotes macrophages to produce IL-8, IL-6 and tumour necrosis factor-alpha in aplastic anaemia. British Journal of Haematology **142**, 109-114.

Gunawardena, N.K., Fujimaki, Y., Aoki, Y., Mishima, N., Ezaki, T., Uni, S., Kimura, E., 2005. Differential effects of diethylcarbamazine, tetracycline and the combination on *Brugia pahangi* adult females in vitro. Parasitol. Int. **54**, 253-259.

Gutierrez, Y., 1984. Diagnostic features of zoonotic filariae in tissue sections. Hum. Pathol. **15**, 514-525.

Gutierrez-Pena, E.J., Knab, J., Buttner, D.W., 1998. Immunoelectron microscopic evidence for release of eosinophil granule matrix protein onto microfilariae of *Onchocerca volvulus* in the skin after exposure to amocarzine. Parasitology Research **84**, 607-615.

Gutierrez-Pena, E.J., Knab, J., Buttner, D.W., 1996. Neutrophil granule proteins: Evidence for the participation in the host reaction to skin microfilariae of *Onchocerca volvulus* after diethylcarbamazine administration. Parasitology **113**, 403-414.

Hafez, I., Stolpe, A., Lindau, M., 2003. Compound exocytosis and cumulative fusion in eosinophils. Journal of Biological Chemistry **278**, 44921-44928.

Hall, L.R., Pearlman, E., 1999. Pathogenesis of onchocercal keratitis (River blindness). Clin. Microbiol. Rev. **12**, 445-453.

Hallen-Sandgren, C., Bjork, I., 1988. A rapid technique for the isolation of highly purified, functionally intact bovine neutrophilic granulocytes. Vet. Immunol. Immunopathol. **18**, 81-94.

Hamann, K.J., Gleich, G.J., Checkel, J.L., Loegering, D.A., McCall, J.W., Barker, R.L., 1990. In vitro killing of microfilariae of *Brugia pahangi* and *Brugia malayi* by eosinophil granule proteins. J. Immunol. **144**, 3166-3173.

Hansch, G.M., Radsak, M., Wagner, C., Reis, B., Koch, A., Breitbart, A., Andrassy, K., 1999. Expression of major histocompatibility class II antigens on polymorphonuclear neutrophils in patients with Wegener's granulomatosis. Kidney Int. **55**, 1811-1818.

Hartog, G., Savelkoul, H.F.J., Schoemaker, R., Tijhaar, E., Westphal, A.H., de Ruiter, T., Weg-Schrijver, E., van Neerven, R.J.J., 2011. Modulation of Human Immune Responses by Bovine Interleukin-10. Plos One **6**, 3, e18188.

Hayashi, F., Means, T.K., Luster, A.D., 2003. Toll-like receptors stimulate human neutrophil function. Blood **102**, 2660-2669.

Hedges, L.M., Brownlie, J.C., O'Neill, S.L., Johnson, K.N., 2008. *Wolbachia* and virus protection in insects. Science **322**, 702.

Heider, U., Blaxter, M., Hoerauf, A., Pfarr, K.M., 2006. Differential display of genes expressed in the filarial nematode *Litomosoides sigmodontis* reveals a putative phosphate permease up-regulated after depletion of *Wolbachia* endobacteria. Int. J. Med. Microbiol. **296**, 287-299.

Heit, B., Tavener, S., Raharjo, E., Kubes, P., 2002. An intracellular signaling hierarchy determines direction of migration in opposing chemotactic gradients. J. Cell Biol. **159**, 91-102.

Henderson, B., Poole, S., Wilson, M., 1996. Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. Microbiol. Rev. **60**, 316-341.

Henrichfreise, B., Schiefer, A., Schneider, T., Nzukou, E., Poellinger, C., Hoffmann, T.J., Johnston, K.L., Moelleken, K., Wiedemann, I., Pfarr, K., Hoerauf, A., Sahl, H.G., 2009. Functional conservation of the lipid II biosynthesis pathway in the cell wall-less bacteria *Chlamydia* and *Wolbachia*: why is lipid II needed? Mol. Microbiol. **73**, 913-923.

Hermans, P.G., Hart, C.A., Trees, A.J., 2001. *In vitro* activity of antimicrobial agents against the endosymbiont *Wolbachia pipientis*. J. Antimicrob. Chemother. **47**, 659-663.

Hertig, M., Wolbach, S.B., 1924. Studies on Rickettsia-Like Micro-Organisms in Insects. J. Med. Res. **44**, 329-374.

Higazi, T.B., Filiano, A., Katholi, C.R., Dadzie, Y., Remme, J.H., Unnasch, T.R., 2005. *Wolbachia* endosymbiont levels in severe and mild strains of Onchocerca volvulus. Mol. Biochem. Parasitol. **141**, 109-112.

Higazi, T.B., Shu, L.M., Unnasch, T.R., 2004. Development and transfection of short-term primary cell cultures from *Brugia malayi*. Molecular and Biochemical Parasitology **137**, 345-348.

Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A., Werren, J.H., 2008. How many species are infected with *Wolbachia*?--A statistical analysis of current data. FEMS Microbiol. Lett. **281**, 215-220.

Hillyer, P., Male, D., 2005. Expression of chemokines on the surface of different human endothelia. Immunol. Cell Biol. **83**, 375-382.

Hiroki, M., Kato, Y., Kamito, T., Miura, K., 2002. Feminization of genetic males by a symbiotic bacterium in a butterfly, Eurema hecabe (Lepidoptera: Pieridae). Naturwissenschaften **89**, 167-170.

Hirschfeld, M., Ma, Y., Weis, J.H., Vogel, S.N., Weis, J.J., 2000. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. J. Immunol. **165**, 618-622.

Hoch, R.C., Schraufstatter, I.U., Cochrane, C.G., 1996. *In vivo*, *in vitro*, and molecular aspects of interleukin-8 and the interleukin-8 receptors. J. Lab Clin. Med. **128**, 134-145.

Hoerauf, A., Brattig, N., 2002. Resistance and susceptibility in human onchocerciasis--beyond Th1 vs. Th2. Trends Parasitol. **18**, 25-31.

Hoerauf, A., Kruse, S., Brattig, N.W., Heinzmann, A., Mueller-Myhsok, B., Deichmann, K.A., 2002. The variant Arg110Gln of human IL-13 is associated with an immunologically hyper-reactive form of onchocerciasis (sowda). Microbes and Infection **4**, 37-42.

Hoerauf, A., Mand, S., Adjei, O., Fleischer, B., Buttner, D.W., 2001. Depletion of *Wolbachia* endobacteria in *Onchocerca volvulus* by doxycycline and microfilaridermia after ivermectin treatment. Lancet **357**, 1415-1416.

Hoerauf, A., Mand, S., Fischer, K., Kruppa, T., Marfo-Debrekyei, Y., Debrah, A.Y., Pfarr, K.M., Adjei, O., Buttner, D.W., 2003a. Doxycycline as a novel strategy against bancroftian filariasis-depletion of *Wolbachia* endosymbionts from *Wuchereria bancrofti* and stop of microfilaria production. Med. Microbiol. Immunol. **192**, 211-216.

Hoerauf, A., Mand, S., Volkmann, L., Buttner, M., Marfo-Debrekyei, Y., Taylor, M., Adjei, O., Buttner, D.W., 2003b. Doxycycline in the treatment of human onchocerciasis: Kinetics of *Wolbachia* endobacteria reduction and of inhibition of embryogenesis in female *Onchocerca* worms. Microbes. Infect. **5**, 261-273.

Hoerauf, A., Nissen-Pahle, K., Schmetz, C., Henkle-Duhrsen, K., Blaxter, M.L., Buttner, D.W., Gallin, M.Y., Al-Qaoud, K.M., Lucius, R., Fleischer, B., 1999. Tetracycline therapy targets intracellular bacteria in the filarial nematode *Litomosoides sigmodontis* and results in filarial infertility. J. Clin. Invest **103**, 11-18.

Hoerauf, A., Specht, S., Buttner, M., Pfarr, K., Mand, S., Fimmers, R., Marfo-Debrekyei, Y., Konadu, P., Debrah, A.Y., Bandi, C., Brattig, N., Albers, A.,

Larbi, J., Batsa, L., Taylor, M.J., Adjei, O., Buttner, D.W., 2008. *Wolbachia* endobacteria depletion by doxycycline as antifilarial therapy has macrofilaricidal activity in onchocerciasis: a randomized placebo-controlled study. Med. Microbiol. Immunol. **197**, 295-311.

Hoerauf, A., Volkmann, L., Hamelmann, C., Adjei, O., Autenrieth, I.B., Fleischer, B., Buttner, D.W., 2000a. Endosymbiotic bacteria in worms as targets for a novel chemotherapy in filariasis. Lancet **355**, 1242-1243.

Hoerauf, A., Volkmann, L., Nissen-Paehle, K., Schmetz, C., Autenrieth, I., Buttner, D.W., Fleischer, B., 2000b. Targeting of *Wolbachia* endobacteria in *Litomosoides sigmodontis*: comparison of tetracyclines with chloramphenicol, macrolides and ciprofloxacin. Trop. Med. Int. Health **5**, 275-279.

Horii, Y., Owhashi, M., Fujita, K., Nakanishi, H., Ishii, A., 1988. A comparative study on eosinophil and neutrophil chemotactic activities of various helminth parasites. Parasitol. Res. **75**, 76-78.

Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X.Y., Fukatsu, T., 2010. *Wolbachia* as a bacteriocyte-associated nutritional mutualist. Proc. Natl. Acad. Sci. U. S. A **107**, 769-774.

Huber, A.R., Kunkel, S.L., Todd, R.F., III, Weiss, S.J., 1991. Regulation of transendothelial neutrophil migration by endogenous interleukin-8. Science 254, 99-102.

Hurst, G.D.D., Jiggins, F.M., von der Schulenburg, J.H.G., Bertrand, D., West, S.A., Goriacheva, I.I., Zakharov, I.A., Werren, J.H., Stouthamer, R., Majerus, M.E.N., 1999. Male-killing *Wolbachia* in two species of insect. Proceedings of the Royal Society of London Series B-Biological Sciences **266**, 735-740.

Hussein, M.R., 2008. Mucocutaneous Splendore-Hoeppli Phenomenon. J. Cutaneous Pathology **35**, 979-988.

Iking-Konert, C., Vogt, S., Radsak, M., Wagner, C., Hansch, G.M., Andrassy, K., 2001. Polymorphonuclear neutrophils in Wegener's granulomatosis acquire characteristics of antigen presenting cells. Kidney International **60**, 2247-2262.

Imaizumi, T., Albertine, K.H., Jicha, D.L., McIntyre, T.M., Prescott, S.M., Zimmerman, G.A., 1997. Human endothelial cells synthesize ENA-78: relationship to IL-8 and to signaling of PMN adhesion. Am. J. Respir. Cell Mol. Biol. **17**, 181-192.

Iwama, T., Nagai, H., Suda, H., Tsuruoka, N., Koda, A., 1992. Effect of murine recombinant interleukin-5 on the cell population in guinea-pig airways. Br. J. Pharmacol. **105**, 19-22.

Iyer, J.K., Coggeshall, K.M., 2011. Cutting edge: primary innate immune cells respond efficiently to polymeric peptidoglycan, but not to peptidoglycan monomers. J. Immunol. **186**, 3841-3845.

Jenson, J.S., O'Connor, R., Osborne, J., Devaney, E., 2002. Infection with Brugia microfilariae induces apoptosis of CD4(+) T lymphocytes: a mechanism of immune unresponsiveness in filariasis. Eur. J. Immunol. **32**, 858-867.

Johnson, E.H., Lustigman, S., Brotman, B., Browne, J., Prince, A.M., 1991. *Onchocerca volvulus*: *in vitro* killing of microfilaria by neutrophils and eosinophils from experimentally infected chimpanzees. Trop. Med. Parasitol. **42**, 351-355.

Johnson, P., Mackenzie, C.D., Suswillo, R.R., Denham, D.A., 1981. Serum-mediated adherence of feline granulocytes to microfilariae of *Brugia pahangi in vitro*: variations with parasite maturation. Parasite Immunol. **3**, 69-80.

Johnston, K.L., Wu, B., Guimaraes, A., Ford, L., Slatko, B.E., Taylor, M.J., 2010. Lipoprotein biosynthesis as a target for anti-*Wolbachia* treatment of filarial nematodes. Parasit. Vectors. **3**, 99.

Jones, D.A., Abbassi, O., McIntire, L.V., McEver, R.P., Smith, C.W., 1993. P-selectin mediates neutrophil rolling on histamine-stimulated endothelial cells. Biophys. J. **65**, 1560-1569.

Juchault, P., Mocquard, J.P., 1993. Transfer of A Parasitic Sex Factor to the Nuclear Genome of the Host - A Hypothesis on the Evolution of Sex-Determining Mechanisms in the Terrestrial Isopod *Armadillidium-Vulgare* Latr. Journal of Evolutionary Biology **6**, 511-528.

Junger, W.G., Cardoza, T.A., Liu, F.C., Hoyt, D.B., Goodwin, R., 1993. Improved rapid photometric assay for quantitative measurement of PMN migration. J. Immunol. Methods **160**, 73-79.

Kadowaki, N., Ho, S., Antonenko, S., Malefyt, R.W., Kastelein, R.A., Bazan, F., Liu, Y.J., 2001. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J. Exp. Med. **194**, 863-869.

Kaifi, J.T., Diaconu, E., Pearlman, E., 2001. Distinct roles for PECAM-1, ICAM-1, and VCAM-1 in recruitment of neutrophils and eosinophils to the cornea in ocular onchocerciasis (river blindness). J. Immunol. **166**, 6795-6801.

Kaiser, W., Huguet, E., Casas, J., Commin, C., Giron, D., 2010. Plant green-island phenotype induced by leaf-miners is mediated by bacterial symbionts. Proc. Biol. Sci. **277**, 2311-2319.

Kambris, Z., Cook, P.E., Phuc, H.K., Sinkins, S.P., 2009. Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. Science **326**, 134-136.

Kazura, J.W., Aikawa, M., 1980. Host defense mechanisms against *Trichinella spiralis* infection in the mouse: eosinophil-mediated destruction of newborn larvae *in vitro*. J. Immunol. **124**, 355-361.

Keiser, P.B., Reynolds, S.M., Awadzi, K., Ottesen, E.A., Taylor, M.J., Nutman, T.B., 2002. Bacterial endosymbionts of *Onchocerca volvulus* in the pathogenesis of posttreatment reactions. J. Infect. Dis. **185**, 805-811.

Kelner, G.S., Kennedy, J., Bacon, K.B., Kleyensteuber, S., Largaespada, D.A., Jenkins, N.A., Copeland, N.G., Bazan, J.F., Moore, K.W., Schall, T.J.,, 1994. Lymphotactin: a cytokine that represents a new class of chemokine. Science **266**, 1395-1399.

Kephart, G.M., Gleich, G.J., Connor, D.H., Gibson, D.W., Ackerman, S.J., 1984a. Deposition of eosinophil granule major basic protein onto microfilariae of *Onchocerca volvulus* in the skin of patients treated with diethylcarbamazine. Lab Invest **50**, 51-61.

Kerepesi, L.A., Leon, O., Lustigman, S., Abraham, D., 2005. Protective immunity to the larval stages of *Onchocerca volvulus* is dependent on Toll-like receptor 4. Infect. Immun. **73**, 8291-8297.

King, C.H., Spagnuolo, P.J., Greene, B.M., 1983. Chemotaxis of human granulocytes toward microfilariae of *Onchocerca volvulus*. Parasite Immunol. **5**, 217-224.

King, C.L., Nutman, T.B., 1991. Regulation of the immune response in lymphatic filariasis and onchocerciasis. Immunol. Today **12**, A54-A58.

Kita, H., Weiler, D.A., Abu-Ghazaleh, R., Sanderson, C.J., Gleich, G.J., 1992. Release of granule proteins from eosinophils cultured with IL-5. J. Immunol. **149**, 629-635.

Klager, S.L., Whitworth, J.A., Downham, M.D., 1996. Viability and fertility of adult *Onchocerca volvulus* after 6 years of treatment with ivermectin. Trop. Med. Int. Health **1**, 581-589.

Klesius, P.H., 1993. Regulation of Immunity to *Ostertagia-Ostertagi*. Veterinary Parasitology **46**, 63-79.

Klion, A.D., Nutman, T.B., 2004. The role of eosinophils in host defense against helminth parasites. J. Allergy Clin. Immunol. **113**, 30-37.

Kogan-Sakin, I., Cohen, M., Paland, N., Madar, S., Solomon, H., Molchadsky, A., Brosh, R., Buganim, Y., Goldfinger, N., Klocker, H., Schalken, J.A., Rotter, V., 2009. Prostate stromal cells produce CXCL-1, CXCL-2, CXCL-3 and IL-8 in response to epithelia-secreted IL-1. Carcinogenesis **30**, 698-705.

Komiyama, A., Spicer, S.S., 1975. Microendocytosis in eosinophilic leukocytes. J. Cell Biol. **64**, 622-635.

Koppenhöfer, H.S and Gaugler, R., 2009. Entomopathogenic nematode and bacteria mutualism. Defensive Mutualism in Microbial Symbiosis. (ed. White, J.F and Torres, M.S). CRC Press **27**, 99-116.

Korten, S., Badusche, M., Buttner, D.W., Hoerauf, A., Brattig, N., Fleischer, B., 2008. Natural death of adult *Onchocerca volvulus* and filaricidal effects of doxycycline induce local FOXP3+/CD4+ regulatory T cells and granzyme expression. Microbes. Infect. **10**, 313-324.

Korten, S., Kaifi, J.T., Buttner, D.W., Hoerauf, A., 2010. Transforming growth factor-beta expression by host cells is elicited locally by the filarial nematode *Onchocerca volvulus* in hyporeactive patients independently from *Wolbachia*. Microbes. Infect. **12**, 555-564.

Korten, S., Wildenburg, G., Darge, K., Buttner, D.W., 1998. Mast cells in onchocercomas from patients with hyperreactive onchocerciasis (sowda). Acta Trop. **70**, 217-231.

Kozek, W.J., 1977. Transovarially-transmitted intracellular microorganisms in adult and larval stages of *Brugia malayi*. J. Parasitol. **63**, 992-1000.

Kozek, W.J., Marroquin, H.F., 1977. Intracytoplasmic bacteria in *Onchocerca volvulus*. Am. J. Trop. Med. Hyg. **26**, 663-678.

Kramer, L., Grandi, G., Leoni, M., Passeri, B., McCall, J., Genchi, C., Mortarino, M., Bazzocchi, C., 2008. *Wolbachia* and its influence on the pathology and immunology of *Dirofilaria immitis* infection. Vet. Parasitol. **158**, 191-195.

Kramer, L., Simon, F., Tamarozzi, F., Genchi, M., Bazzocchi, C., 2005. Is *Wolbachia* complicating the pathological effects of *Dirofilaria immitis* infections? Vet. Parasitol. **133**, 133-136.

Kramer, L.H., Passeri, B., Corona, S., Simoncini, L., Casiraghi, M., 2003. Immunohistochemical/immunogold detection and distribution of the endosymbiont *Wolbachia* of *Dirofilaria immitis* and *Brugia pahangi* using a polyclonal antiserum raised against WSP (*Wolbachia* surface protein). Parasitology Research **89**, 381-386.

Krause, S., Sommer, A., Fischer, P., Brophy, P.M., Walter, R.D., Liebau, E., 2001. Gene structure of the extracellular glutathione S-transferase from *Onchocerca volvulus* and its overexpression and promoter analysis in transgenic *Caenorhabditis elegans*. Molecular and Biochemical Parasitology **117**, 145-154.

Krueger, A., Fischer, P., Morales-Hojas, R., 2007. Molecular phylogeny of the filaria genus *Onchocerca* with special emphasis on Afrotropical human and bovine parasites. Acta Tropica **101**, 1-14.

Kurt-Jones, E.A., Mandell, L., Whitney, C., Padgett, A., Gosselin, K., Newburger, P.E., Finberg, R.W., 2002. Role of toll-like receptor 2 (TLR2) in neutrophil

activation: GM-CSF enhances TLR2 expression and TLR2-mediated interleukin 8 responses in neutrophils. Blood **100**, 1860-1868.

Kvietys, P.R., Sandig, M., 2001. Neutrophil diapedesis: paracellular or transcellular? News Physiol Sci. **16**, 15-19.

Kwong, L.S., Hope, J.C., Thom, M.L., Sopp, P., Duggan, S., Bembridge, G.P., Howard, C.J., 2002. Development of an ELISA for bovine IL-10. Veterinary Immunology and Immunopathology **85**, 213-223.

Lamote, I., Demeyere, K., Notebaert, S., Burvenich, C., Meyer, E., 2007. Flow cytometric assessment of estrogen receptor beta expression in bovine blood neutrophils. Journal of Immunological Methods **323**, 88-92.

Landmann, F., Foster, J.M., Slatko, B., Sullivan, W., 2010. Asymmetric *Wolbachia* segregation during early *Brugia malayi* embryogenesis determines its distribution in adult host tissues. PLoS Negl. Trop. Dis. **4**, e758.

Lange, A.M., Yutanawiboonchai, W., Scott, P., Abraham, D., 1994. IL-4- and IL-5-dependent protective immunity to *Onchocerca volvulus* infective larvae in BALB/cBYJ mice. J. Immunol. **153**, 205-211.

Langworthy, N.G., Renz, A., Mackenstedt, U., Henkle-Duhrsen, K., de Bronsvoort, M.B., Tanya, V.N., Donnelly, M.J., Trees, A.J., 2000. Macrofilaricidal activity of tetracycline against the filarial nematode *Onchocerca ochengi*: elimination of *Wolbachia* precedes worm death and suggests a dependent relationship. Proc. Biol. Sci. **267**, 1063-1069.

Laudanna, C., Kim, J.Y., Constantin, G., Butcher, E.C., 2002. Rapid leukocyte integrin activation by chemokines. Immunological Reviews **186**, 37-46.

Lawrence, M.B., Bainton, D.F., Springer, T.A., 1994. Neutrophil tethering to and rolling on E-selectin are separable by requirement for L-selectin. Immunity. **1**, 137-145.

Le Goff G.L., Loke, P., Ali, H.F., Taylor, D.W., Allen, J.E., 2000a. Interleukin-5 is essential for vaccine-mediated immunity but not innate resistance to a filarial parasite. Infect. Immun. **68**, 2513-2517.

Le Goff G.L., Martin, C., Oswald, I.P., Vuong, P.N., Petit, G., Ungeheuer, M.N., Bain, O., 2000b. Parasitology and immunology of mice vaccinated with irradiated *Litomosoides sigmodontis* larvae. Parasitology **120** (Pt 3), 271-280.

Lehrer, R.I., Szklarek, D., Barton, A., Ganz, T., Hamann, K.J., Gleich, G.J., 1989. Antibacterial properties of eosinophil major basic protein and eosinophil cationic protein. J. Immunol. **142**, 4428-4434.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., Hoffmann, J.A., 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. Cell **86**, 973-983.

Liebau, E., Eschbach, M.L., Tawe, W., Sommer, A., Fischer, P., Walter, R.D., Henkle-Duhrsen, K., 2000. Identification of a stress-responsive *Onchocerca volvulus* glutathione S-transferase (*Ov*-GST-3) by RT-PCR differential display. Mol. Biochem. Parasitol. **109**, 101-110.

Limaye, A.P., Abrams, J.S., Silver, J.E., Awadzi, K., Francis, H.F., Ottesen, E.A., Nutman, T.B., 1991. Interleukin-5 and the posttreatment eosinophilia in patients with onchocerciasis. J. Clin. Invest **88**, 1418-1421.

Lu, W., Egerton, G.L., Bianco, A.E., Williams, S.A., 1998. Thioredoxin peroxidase from *Onchocerca volvulus*: a major hydrogen peroxide detoxifying enzyme in filarial parasites. Mol. Biochem. Parasitol. **91**, 221-235.

Lukacs, N.W., Hogaboam, C.M., Kunkel, S.L., Chensue, S.W., Burdick, M.D., Evanoff, H.L., Strieter, R.M., 1998a. Mast cells produce ENA-78, which can function as a potent neutrophil chemoattractant during allergic airway inflammation. J. Leukoc. Biol. **63**, 746-751.

Lumsden, J.H., Mullen, K., Rowe, R., 1980. Hematology and biochemistry reference values for female Holstein cattle. Can. J. Comp Med. **44**, 24-31.

Lund, J.M., Alexopoulou, L., Sato, A., Karow, M., Adams, N.C., Gale, N.W., Akiko, I., Flavell, R.A., 2004. Recognition of single-stranded RNA viruses by Toll-like receptor 7. Proc Natl Acad Sci. **101**(15), 5598–5603.

Lustigman, S., Brotman, B., Huima, T., Prince, A.M., McKerrow, J.H., 1992. Molecular cloning and characterization of onchocystatin, a cysteine proteinase inhibitor of *Onchocerca volvulus*. J. Biol. Chem. **267**, 17339-17346.

Lustigman, S., Huima, T., Brotman, B., Miller, K., Prince, A.M., 1990. *Onchocerca volvulus*: biochemical and morphological characteristics of the surface of third- and fourth-stage larvae. Exp. Parasitol. **71**, 489-495.

Macdonald, A.S., Araujo, M.I., Pearce, E.J., 2002. Immunology of parasitic helminth infections. Infection and Immunity **70**, 427-433.

Maizels, R.M., Bundy, D.A., Selkirk, M.E., Smith, D.F., Anderson, R.M., 1993. Immunological modulation and evasion by helminth parasites in human populations. Nature **365**, 797-805.

Maizels, R.M., Gomez-Escobar, N., Gregory, W.F., Murray, J., Zang, X.X., 2001. Immune evasion genes from filarial nematodes. International Journal for Parasitology **31**, 889-898.

Maizels, R.M., Yazdanbakhsh, M., 2003. Immune regulation by helminth parasites: cellular and molecular mechanisms. Nat. Rev. Immunol. **3**, 733-744.

Makepeace, B.L., Rodgers, L., Trees, A.J., 2006. Rate of elimination of *Wolbachia* pipientis by doxycycline in vitro increases following drug withdrawal. Antimicrob. Agents Chemother. **50**, 922-927.

Mansson, A., Cardell, L., 2007. Toll-like receptors (TLRs) in human eosinophils: link between viral infections and asthma? Allergy **62**, 439.

Martin, C., Al-Qaoud, K.M., Ungeheuer, M.N., Paehle, K., Vuong, P.N., Bain, O., Fleischer, B., Hoerauf, A., 2000a. IL-5 is essential for vaccine-induced protection and for resolution of primary infection in murine filariasis. Med. Microbiol. Immunol. **189**, 67-74.

Martin, C., Le, G.L., Ungeheuer, M.N., Vuong, P.N., Bain, O., 2000b. Drastic reduction of a filarial infection in eosinophilic interleukin-5 transgenic mice. Infect. Immun. **68**, 3651-3656.

Mathers, C.D., Ezzati, M., Lopez, A.D., 2007. Measuring the burden of neglected tropical diseases: the global burden of disease framework. PLoS. Negl. Trop. Dis. 1, (2), e114.

Matsuba-Kitamura, S., Yoshimoto, T., Yasuda, K., Futatsugi-Yumikura, S., Taki, Y., Muto, T., Ikeda, T., Mimura, O., Nakanishi, K., 2010. Contribution of IL-33 to induction and augmentation of experimental allergic conjunctivitis. International Immunology **22**, 479-489.

Mattsson, E., Persson, T., Andersson, P., Rollof, J., Egesten, A., 2003. Peptidoglycan induces mobilization of the surface marker for activation marker CD66b in human neutrophils but not in eosinophils. Clin. Diagn. Lab Immunol. **10**, 485-488.

Mayr, S.I., Zuberi, R.I., Zhang, M., de Sousa-Hitzler, J., Ngo, K., Kuwabara, Y., Yu, L., Fung-Leung, W.P., Liu, F.T., 2002. IgE-dependent mast cell activation potentiates airway responses in murine asthma models. J. Immunol. **169**, 2061-2068.

McDonald, P.P., Pouliot, M., Borgeat, P., McColl, S.R., 1993. Induction by chemokines of lipid mediator synthesis in granulocyte-macrophage colony-stimulating factor-treated human neutrophils. J. Immunol. **151**, 6399-6409.

McGarry, H.F., Egerton, G.L., Taylor, M.J., 2004. Population dynamics of *Wolbachia* bacterial endosymbionts in *Brugia malayi*. Mol. Biochem. Parasitol. **135**, 57-67.

McKenzie, G.J., Bancroft, A., Grencis, R.K., McKenzie, A.N.J., 1998. A distinct role for interleukin-13 in Th2-cell-mediated immune responses. Current Biology **8**, 339-342.

McLaren, D.J., McKean, J.R., Olsson, I., Venges, P., Kay, A.B., 1981. Morphological studies on the killing of schistosomula of *Schistosoma mansoni* by human eosinophil and neutrophil cationic proteins in vitro. Parasite Immunol. **3**, 359-373.

McLaren, D.J., Worms, M.J., Laurence, B.R., Simpson, M.G., 1975. Micro-organisms in filarial larvae (Nematoda). Trans. R. Soc. Trop. Med. Hyg. **69**, 509-514.

McNulty, S.N., Foster, J.M., Mitreva, M., Dunning Hotopp, J.C., Martin, J., Fischer, K., Wu, B., Davis, P.J., Kumar, S., Brattig, N.W., Slatko, B.E., Weil, G.J., Fischer, P.U., 2010. Endosymbiont DNA in endobacteria-free filarial nematodes indicates ancient horizontal genetic transfer. PLoS. One. **5**, e11029.

Medina-De la Garza CE, Brattig, N.W., Tischendorf, F.W., Jarrett, J.M., 1990. Serum-dependent interaction of granulocytes with *Onchocerca volvulus* microfilariae in generalized and chronic hyper-reactive onchocerciasis and its modulation by diethylcarbamazine. Trans. R. Soc. Trop. Med. Hyg. **84**, 701-706.

Meeusen, E.N., Balic, A., 2000. Do eosinophils have a role in the killing of helminth parasites? Parasitol. Today **16**, 95-101.

Melnikow, E., Xu, S., Liu, J., Li, L., Oksov, Y., Ghedin, E., Unnasch, T.R., Lustigman, S., 2011. Interaction of a *Wolbachia* WSP-like protein with a nuclear-encoded protein of *Brugia malayi*. Int. J. Parasitol. **41**, 1053-1061.

Menzies, M., Ingham, A., 2006. Identification and expression of Toll-like receptors 1-10 in selected bovine and ovine tissues. Vet. Immunol. Immunopathol. **109**, 23-30.

Mertens, B., Gobright, E., Seow, H.F., 1996. The nucleotide sequence of the bovine interleukin-5-encoding cDNA. Gene **176**, 273-274.

Miller, F., de, H.E., Palade, G.E., 1966. The structure of eosinophil leukocyte granules in rodents and in man. J. Cell Biol. **31**, 349-362.

Miller, W.J., Ehrman, L., Schneider, D., 2010. Infectious speciation revisited: impact of symbiont-depletion on female fitness and mating behavior of *Drosophila paulistorum*. PLoS Pathog. **6**, e1001214.

Mitchell, G.B., Albright, B.N., Caswell, J.L., 2003. Effect of interleukin-8 and granulocyte colony-stimulating factor on priming and activation of bovine neutrophils. Infect. Immun. **71**, 1643-1649.

Modi, W.S., Yoshimura, T., 1999. Isolation of novel GRO genes and a phylogenetic analysis of the CXC chemokine subfamily in mammals. Mol. Biol. Evol. **16**, 180-193.

Moqbel, R., Coughlin, J.J., 2006. Differential secretion of cytokines. Sci. STKE. 2006, (338) pe26.

Morchon, R., Bazzocchi, C., Lopez-Belmonte, J., Martin-Pacho, J.R., Kramer, L.H., Grandi, G., Simon, F., 2007. iNOs expression is stimulated by the major surface protein (rWSP) from *Wolbachia* bacterial endosymbiont of *Dirofilaria immitis* following subcutaneous injection in mice. Parasitol. Int. **56**, 71-75.

Moreira, L.A., Iturbe-Ormaetxe, I., Jeffery, J.A., Lu, G., Pyke, A.T., Hedges, L.M., Rocha, B.C., Hall-Mendelin, S., Day, A., Riegler, M., Hugo, L.E., Johnson,

K.N., Kay, B.H., McGraw, E.A., van den Hurk, A.F., Ryan, P.A., O'Neill, S.L., 2009. A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and Plasmodium. Cell **139**, 1268-1278.

Moreno, Y., Nabhan, J.F., Solomon, J., Mackenzie, C.D., Geary, T.G., 2010. Ivermectin disrupts the function of the excretory-secretory apparatus in microfilariae of *Brugia malayi*. Proc. Natl. Acad. Sci. U. S. A **107**, 20120-20125.

Muller, S., Walter, R.D., Fairlamb, A.H., 1995. Differential susceptibility of filarial and human erythrocyte glutathione reductase to inhibition by the trivalent organic arsenical melarsen oxide. Mol. Biochem. Parasitol. **71**, 211-219.

Murdoch, M.E., Hay, R.J., Mackenzie, C.D., Williams, J.F., Ghalib, H.W., Cousens, S., Abiose, A., Jones, B.R., 1993. A clinical classification and grading system of the cutaneous changes in onchocerciasis. Br. J. Dermatol. **129**, 260-269.

Musher, D.M., Verbrugh, H.A., Verhoef, J., 1981. Suppression of phagocytosis and chemotaxis by cell wall components of *Staphylococcus aureus*. J. Immunol. **127**, 84-88.

Nagase, H., Okugawa, S., Ota, Y., Yamaguchi, M., Tomizawa, H., Matsushima, K., Ohta, K., Yamamoto, K., Hirai, K., 2003. Expression and function of Toll-like receptors in eosinophils: activation by Toll-like receptor 7 ligand. J. Immunol. **171**, 3977-3982.

Nakao, Y., Funami, K., Kikkawa, S., Taniguchi, M., Nishiguchi, M., Fukumori, Y., Seya, T., Matsumoto, M., 2005. Surface-expressed TLR6 participates in the recognition of diacylated lipopeptide and peptidoglycan in human cells. J. Immunol. **174**, 1566-1573.

Neary, J.M., Trees, A.J., Ekale, D.D., Tanya, V.N., Hetzel, U., Makepeace, B.L., 2010. *Onchocerca armillata* contains the endosymbiotic bacterium *Wolbachia* and elicits a limited inflammatory response. Vet. Parasitol. **174**, 267-276.

Nfon, C.K., Makepeace, B.L., Njongmeta, L.M., Tanya, V.N., Bain, O., Trees, A.J., 2006. Eosinophils contribute to killing of adult *Onchocerca ochengi* within onchocercomata following elimination of *Wolbachia*. Microbes. Infect. **8**, 2698-2705.

Njoo, F.L., Hack, C.E., Oosting, J., Stilma, J.S., Kijlstra, A., 1993. Neutrophil activation in ivermectin-treated onchocerciasis patients. Clin. Exp. Immunol. **94**, 330-333.

Noroes, J., Dreyer, G., Santos, A., Mendes, V.G., Medeiros, Z., Addiss, D., 1997. Assessment of the efficacy of diethylcarbamazine on adult *Wuchereria bancrofti* in vivo. Trans. R. Soc. Trop. Med. Hyg. **91**, 78-81.

Numasaki, M., Lotze, M.T., Sasaki, H., 2004. Interleukin-17 augments tumor necrosis factor-alpha-induced elaboration of proangiogenic factors from fibroblasts. Immunol. Lett. **93**, 39-43.

O'Mahony, D.S., Pham, U., Iyer, R., Hawn, T.R., Liles, W.C., 2008. Differential constitutive and cytokine-modulated expression of human Toll-like receptors in primary neutrophils, monocytes, and macrophages. Int. J. Med. Sci. **5**, 1-8.

O'Neill, S.L., Pettigrew, M.M., Sinkins, S.P., Braig, H.R., Andreadis, T.G., Tesh, R.B., 1997. In vitro cultivation of *Wolbachia pipientis* in an *Aedes albopictus* cell line. Insect Mol. Biol. **6**, 33-39.

Osborne, S.E., Leong, Y.S., O'Neill, S.L., Johnson, K.N., 2009. Variation in antiviral protection mediated by different *Wolbachia* strains in *Drosophila simulans*. PLoS Pathog. **5**, e1000656.

Osei-Atweneboana, M.Y., Awadzi, K., Attah, S.K., Boakye, D.A., Gyapong, J.O., Prichard, R.K., 2011. Phenotypic evidence of emerging ivermectin resistance in *Onchocerca volvulus*. PLoS. Negl. Trop. Dis. **5**, e998.

Ottesen, E.A., 1995. Immune responsiveness and the pathogenesis of human onchocerciasis. J. Infect. Dis. **171**, 659-671.

Ouyang, W., Kolls, J.K., Zheng, Y., 2008. The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity. **28**, 454-467.

Paape, M.J., Bannerman, D.D., Zhao, X., Lee, J.W., 2003. The bovine neutrophil: Structure and function in blood and milk. Vet. Res. **34**, 597-627.

Padgett, G.A., Hirsch, J.G., 1967. Lysozyme: its absence in tears and leukocytes of cattle. Aust. J. Exp. Biol. Med. Sci. **45**, 569-570.

Palframan, R.T., Collins, P.D., Williams, T.J., Rankin, S.M., 1998. Eotaxin induces a rapid release of eosinophils and their progenitors from the bone marrow. Blood **91**, 2240-2248.

Parmley, R.T., Spicer, S.S., 1975. Altered tissue eosinophils in Hodgkin's Disease. Exp. Mol. Pathol. **23**, 70-82.

Pastrana, D.V., Raghavan, N., FitzGerald, P., Eisinger, S.W., Metz, C., Bucala, R., Schleimer, R.P., Bickel, C., Scott, A.L., 1998. Filarial nematode parasites secrete a homologue of the human cytokine macrophage migration inhibitory factor. Infect. Immun. **66**, 5955-5963.

Pearlman, E., Toe, L., Boatin, B.A., Gilles, A.A., Higgins, A.W., Unnasch, T.R., 1999. Eotaxin expression in *Onchocerca volvulus*-induced dermatitis after topical application of diethylcarbamazine. J. Infect. Dis. **180**, 1394-1397.

Pelletier, M., Maggi, L., Micheletti, A., Lazzeri, E., Tamassia, N., Costantini, C., Cosmi, L., Lunardi, C., Annunziato, F., Romagnani, S., Cassatella, M.A., 2010. Evidence for a cross-talk between human neutrophils and Th17 cells. Blood **115**, 335-343.

Pellme, S., Morgelin, M., Tapper, H., Mellqvist, U.H., Dahlgren, C., Karlsson, A., 2006. Localization of human neutrophil interleukin-8 (CXCL-8) to organelle(s) distinct from the classical granules and secretory vesicles. Journal of Leukocyte Biology **79**, 564-573.

Pemberton, K.D., Barrett, J., 1989. The detoxification of xenobiotic compounds by *Onchocerca gutturosa* (Nematoda: Filarioidea). Int. J. Parasitol. **19**, 875-878.

Persson, T., Monsef, N., Andersson, P., Bjartell, A., Malm, J., Calafat, J., Egesten, A., 2003. Expression of the neutrophil-activating CXC chemokine ENA-78/CXCL5 by human eosinophils. Clin. Exp. Allergy **33**, 531-537.

Peveri, P., Walz, A., Dewald, B., Baggiolini, M., 1988. A novel neutrophil-activating factor produced by human mononuclear phagocytes. J. Exp. Med. **167**, 1547-1559.

Phipps, S., Lam, C.E., Mahalingam, S., Newhouse, M., Ramirez, R., Rosenberg, H.F., Foster, P.S., Matthaei, K.I., 2007. Eosinophils contribute to innate antiviral immunity and promote clearance of respiratory syncytial virus. Blood **110**, 1578-1586.

Phipps, S., Ying, S., Wangoo, A., Ong, Y.E., Levi-Schaffer, F., Kay, A.B., 2002. The relationship between allergen-induced tissue eosinophilia and markers of repair and remodeling in human atopic skin. J. Immunol. **169**, 4604-4612.

Piessens, W.F., Beldekas, M., 1979. Diethylcarbamazine enhances antibody-mediated cellular adherence to *Brugia malayi* microfilariae. Nature **282**, 845-847.

Pintureau, B., Chapelle, L., Delobel, B., 1999. Effects of repeated thermic and antibiotic treatments on a *Trichogramma* (Hym., Trichogrammatidae) symbiont. Journal of Applied Entomology-Zeitschrift fur Angewandte Entomologie **123**, 473-483.

Plenge-Bonig, A., Kromer, M., Buttner, D.W., 1995. Light and electron microscopy studies on *Onchocerca jakutensis* and *O. flexuosa* of red deer show different host-parasite interactions. Parasitol. Res. **81**, 66-73.

Racz, P., Tenner-Racz, K., Buttner, D.W., Albiez, E.J., 1982. Ultrastructural evidence for eosinophil-parasite adherence (EPA) reaction in human onchocercal lymphadenitis in the early period following diethylcarbamazine treatment. Tropenmed. Parasitol. **33**, 213-218.

Rainard, P., Riollet, C., Berthon, P., Cunha, P., Fromageau, A., Rossignol, C., Gilbert, F.B., 2008. The chemokine CXCL3 is responsible for the constitutive chemotactic activity of bovine milk for neutrophils. Mol. Immunol. **45**, 4020-4027.

Rainard, P., Sarradin, P., Paape, M.J., Poutrel, B., 1998. Quantification of C5a/C5a(desArg) in bovine plasma, serum and milk. Vet. Res. **29**, 73-88.

Rainbird, M.A., Macmillan, D., Meeusen, E.N., 1998. Eosinophil-mediated killing of *Haemonchus contortus* larvae: effect of eosinophil activation and role of antibody, complement and interleukin-5. Parasite Immunol. **20**, 93-103.

Rajan, T.V., 2004. Relationship of anti-microbial activity of tetracyclines to their ability to block the L3 to L4 molt of the human filarial parasite *Brugia malayi*. Am. J. Trop. Med. Hyg. **71**, 24-28.

Ramos, A.L., Discipio, R.G., Ferreira, A.M., 2006. Eosinophil cationic protein damages protoscoleces *in vitro* and is present in the hydatid cyst. Parasite Immunol. **28**, 347-355.

Rankin, S.M., Conroy, D.M., Williams, T.J., 2000. Eotaxin and eosinophil recruitment: implications for human disease. Mol. Med. Today **6**, 20-27.

Rao, R., Well, G.J., 2002. In vitro effects of antibiotics on *Brugia malayi* worm survival and reproduction. J. Parasitol. **88**, 605-611.

Rao, R.U., Klei, T.R., 2006. Cytokine profiles of filarial granulomas in jirds infected with *Brugia pahangi*. Filaria. J. **5**, 3.

Rausch, P.G., Moore, T.G., 1975. Granule enzymes of polymorphonuclear neutrophils: A phylogenetic comparison. Blood **46**, 913-919.

Reese, T.A., Liang, H.E., Tager, A.M., Luster, A.D., Van, R.N., Voehringer, D., Locksley, R.M., 2007. Chitin induces accumulation in tissue of innate immune cells associated with allergy. Nature **447**, 92-96.

Remme, J.H., 2004. Research for control: the onchocerciasis experience. Trop. Med. Int. Health **9**, 243-254.

Renz, A., Trees, A.J., Achu-Kwi, D., Edwards, G., Wahl, G., 1995. Evaluation of suramin, ivermectin and CGP 20376 in a new macrofilaricidal drug screen, *Onchocerca ochengi* in African cattle. Trop. Med. Parasitol. **46**, 31-37.

Riber, U., Espersen, F., Wilkinson, B.J., Kharazmi, A., 1990. Neutrophil chemotactic activity of peptidoglycan. A comparison between *Staphylococcus aureus* and *Staphylococcus epidermidis*. APMIS **98**, 881-886.

Richards, F.O., Jr., Amann, J., Arana, B., Punkosdy, G., Klein, R., Blanco, C., Lopez, B., Mendoza, C., Dominguez, A., Guarner, J., Maguire, J.H., Eberhard, M., 2007. No depletion of *Wolbachia* from *Onchocerca volvulus* after a short course of rifampin and/or azithromycin. Am. J. Trop. Med. Hyg. **77**, 878-882.

Riding, G.A., Willadsen, P., 1981. Simultaneous isolation of bovine eosinophils and neutrophils on gradients of Percoll. J. Immunol. Methods **46**, 113-119.

Riethmiller, S., 2005. From Atoxyl to Salvarsan: Searching for the magic bullet. Chemotherapy **51**, 234-242.

Rockey, J.H., John, T., Donnelly, J.J., McKenzie, D.F., Stromberg, B.E., Soulsby, E.J., 1983. *In vitro* interaction of eosinophils from ascarid-infected eyes with *Ascaris suum* and *Toxocara canis* larvae. Invest Ophthalmol. Vis. Sci. **24**, 1346-1357.

Rollinson, D., Hay, S.I., 2008. Advances in Parasitology. Elsevier Academic Press **66**, 218-219

Rosbottom, A., Guy, C.S., Gibney, E.H., Smith, R.F., Valarcher, J.F., Taylor, G., Williams, D.J.L., 2007. Peripheral immune responses in pregnant cattle following *Neospora caninum* infection. Parasite Immunology **29**, 219-228.

Roth, J.A., Kaeberle, M.L., 1981. Isolation of neutrophils and eosinophils from the peripheral blood of cattle and comparison of their functional activities. J. Immunol. Methods **45**, 153-164.

Rothenberg, M.E., Hogan, S.P., 2006. The eosinophil. Annu. Rev. Immunol. **24**, 147-174.

Rothenberg, M.E., Luster, A.D., Leder, P., 1995. Murine eotaxin: an eosinophil chemoattractant inducible in endothelial cells and in interleukin 4-induced tumor suppression. Proc. Natl. Acad. Sci. U. S. A **92**, 8960-8964.

Rotman, H.L., Yutanawiboonchai, W., Brigandi, R.A., Leon, O., Gleich, G.J., Nolan, T.J., Schad, G.A., Abraham, D., 1996. *Strongyloides stercoralis*: eosinophil-dependent immune-mediated killing of third stage larvae in BALB/cByJ mice. Exp. Parasitol. **82**, 267-278.

Rubio de Kromer, M.T., Kromer, M., Luersen, K., Brattig, N.W., 1998. Detection of a chemotactic factor for neutrophils in extracts of female *Onchocerca volvulus*. Acta Trop. **71**, 45-56.

Rubio de Kromer, M.T., Medina-De la Garza CE, Brattig, N.W., 1995. Differences in eosinophil and neutrophil chemotactic responses in sowda and generalized form of onchocerciasis. Acta Trop. **60**, 21-33.

Ryu, J.S., Kang, J.H., Jung, S.Y., Shin, M.H., Kim, J.M., Park, H., Min, D.Y., 2004. Production of interleukin-8 by human neutrophils stimulated with *Trichomonas vaginalis*. Infect. Immun. **72**, 1326-1332.

Saeftel, M., Volkmann, L., Korten, S., Brattig, N., Al-Qaoud, K., Fleischer, B., Hoerauf, A., 2001. Lack of interferon-gamma confers impaired neutrophil granulocyte function and imparts prolonged survival of adult filarial worms in murine filariasis. Microbes. Infect. **3**, 203-213.

Saint, A.A., Blackwell, N.M., Hall, L.R., Hoerauf, A., Brattig, N.W., Volkmann, L., Taylor, M.J., Ford, L., Hise, A.G., Lass, J.H., Diaconu, E., Pearlman, E., 2002. The role of endosymbiotic *Wolbachia* bacteria in the pathogenesis of river blindness. Science **295**, 1892-1895.

Satoguina, J., Mempel, M., Larbi, J., Badusche, M., Loliger, C., Adjei, O., Gachelin, G., Fleischer, B., Hoerauf, A., 2002. Antigen-specific T regulatory-1 cells are

associated with immunosuppression in a chronic helminth infection (onchocerciasis). Microbes. Infect. **4**, 1291-1300.

Schiffmann, E., Corcoran, B.A., Wahl, S.M., 1975. N-formylmethionyl peptides as chemoattractants for leucocytes. Proc. Natl. Acad. Sci. U. S. A **72**, 1059-1062.

Schnyder-Candrian, S., Walz, A., 1997. Neutrophil-activating protein ENA-78 and IL-8 exhibit different patterns of expression in lipopolysaccharide- and cytokine-stimulated human monocytes. J. Immunol. **158**, 3888-3894.

Schratl, P., Sturm, E.M., Royer, J.F., Sturm, G.J., Lippe, I.T., Peskar, B.A., Heinemann, A., 2006. Hierarchy of eosinophil chemoattractants: role of p38 mitogen-activated protein kinase. Eur. J. Immunol. **36**, 2401-2409.

Schulz-Key, H., 1988. The collagenase technique: how to isolate and examine adult *Onchocerca volvulus* for the evaluation of drug effects. Trop. Med. Parasitol. **39** Suppl 4, 423-440.

Schweizer, R.C., Welmers, B.A., Raaijmakers, J.A., Zanen, P., Lammers, J.W., Koenderman, L., 1994. RA. Blood **83**, 3697-3704.

Selsted, M.E., Novotny, M.J., Morris, W.L., Tang, Y.Q., Smith, W., Cullor, J.S., 1992. Indolicidin, a novel bactericidal tridecapeptide amide from neutrophils. J. Biol. Chem. **267**, 4292-4295.

Seow, H.F., Yoshimura, T., Wood, P.R., Colditz, I.G., 1994. Cloning, sequencing, expression and inflammatory activity in skin of ovine interleukin-8. Immunol. Cell Biol. **72**, 398-405.

Shaik, S.S., Soltau, T.D., Chaturvedi, G., Totapally, B., Hagood, J.S., Andrews, W.W., Athar, M., Voitenok, N.N., Killingsworth, C.R., Patel, R.P., Fallon, M.B., Maheshwari, A., 2009. Low intensity shear stress increases endothelial ELR+ CXC chemokine production via a focal adhesion kinase-p38{beta} MAPK-NF-{kappa}B pathway. J. Biol. Chem. **284**, 5945-5955.

Shiny, C., Krushna, N.S., Babu, S., Elango, S., Manokaran, G., Narayanan, R.B., 2011a. Recombinant *Wolbachia* heat shock protein 60 (HSP60) mediated immune responses in patients with lymphatic filariasis. Microbes Infect.**DOI 10.1016/j.mic.inf.2011.07.004**.

Shiny, C., Krushna, N.S., Haripriya, K., Babu, S., Elango, S., Manokaran, G., Narayanan, R.B., 2011b. Recombinant *Wolbachia* surface protein (WSP)-induced T cell responses in *Wuchereria bancrofti* infections. Parasitol. Res. **DOI 10.1007/s00436-011-2553-7**.

Shuster, D.E., Kehrli, M.E., Rainard, P., Paape, M., 1997. Complement fragment C5a and inflammatory cytokines in neutrophil recruitment during intramammary infection with Escherichia coli. Infection and Immunity **65**, 3286-3292.

Simons, J.E., Rothenberg, M.E., Lawrence, R.A., 2005. Eotaxin-1-regulated eosinophils have a critical role in innate immunity against experimental *Brugia malayi* infection. European Journal of Immunology **35**, 189-197.

Sironi, M., Bandi, C., Sacchi, L., Di, S.B., Damiani, G., Genchi, C., 1995. Molecular evidence for a close relative of the arthropod endosymbiont *Wolbachia* in a filarial worm. Mol. Biochem. Parasitol. **74**, 223-227.

Slatko, B.E., Taylor, M.J., Foster, J.M., 2010. The *Wolbachia* endosymbiont as an anti-filarial nematode target. Symbiosis. **51**, 55-65.

Smith, H.L., Rajan, T.V., 2000. Tetracycline inhibits development of the infective-stage larvae of filarial nematodes in vitro. Exp. Parasitol. **95**, 265-270.

Smith, R.J., Cotter, T.P., Williams, J.F., Guderian, R.H., 1988. Vascular perfusion of *Onchocerca volvulus* nodules. Trop. Med. Parasitol. 39 Suppl **4**, 418-421.

Smith, V.P., Selkirk, M.E., Gounaris, K., 1998. *Brugia malayi*: Resistance of cuticular lipids to oxidant-induced damage and detection of alpha-tocopherol in the neutral lipid fraction. Experimental Parasitology **88**, 103-110.

Soboslay, P.T., Hamm, D.M., Pfafflin, F., Fendt, J., Banla, M., Schulz-Key, H., 2006. Cytokine and chemokine responses in patients co-infected with *Entamoeba histolytica*/dispar, *Necator americanus* and *Mansonella perstans* and changes after anti-parasite treatment. Microbes and Infection **8**, 238-247.

Soldan, A., 1999. Blood transfusions in cattle. In Practice **21**, 590-595.

Soltys, J., Swain, S.D., Sipes, K.M., Nelson, L.K., Hanson, A.J., Kantele, J.M., Jutila, M.A., Quinn, M.T., 1999. Isolation of bovine neutrophils with biomagnetic beads: comparison with standard Percoll density gradient isolation methods. J. Immunol. Methods **226**, 71-84.

Specht, S., Frank, J.K., Alferink, J., Dubben, B., Layland, L.E., Denece, G., Bain, O., Forster, I., Kirschning, C.J., Martin, C., Hoerauf, A., 2011. CCL17 controls mast cells for the defense against filarial larval entry. J. Immunol. **186**, 4845-4852.

Specht, S., Mand, S., Marfo-Debrekyei, Y., Debrah, A.Y., Konadu, P., Adjei, O., Buttner, D.W., Hoerauf, A., 2008. Efficacy of 2- and 4-week rifampicin treatment on the *Wolbachia* of *Onchocerca volvulus*. Parasitol. Res. **103**, 1303-1309.

Specht, S., Saeftel, M., Arndt, M., Endl, E., Dubben, B., Lee, N.A., Lee, J.J., Hoerauf, A., 2006. Lack of eosinophil peroxidase or major basic protein impairs defense against murine filarial infection. Infect. Immun. **74**, 5236-5243.

Springer, T.A., 1990. Adhesion receptors of the immune system. Nature **346**, 425-434.

Springer, T.A., 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell **76**, 301-314.

Stein, L.H., Redding, K.M., Lee, J.J., Nolan, T.J., Schad, G.A., Lok, J.B., Abraham, D., 2009. Eosinophils utilize multiple chemokine receptors for chemotaxis to the parasitic nematode *Strongyloides stercoralis*. J. Innate. Immun. **1**, 618-630.

Stern, M., Meagher, L., Savill, J., Haslett, C., 1992. Apoptosis in human eosinophils. Programmed cell death in the eosinophil leads to phagocytosis by macrophages and is modulated by IL-5. J. Immunol. **148**, 3543-3549.

Stevens, M.G., Van, P.M., Peelman, L.J., Rainard, P., De, S.B., Rogiers, C., Van De Walle, G.R., Duchateau, L., Burvenich, C., 2011. Anaphylatoxin C5a-induced toll-like receptor 4 signaling in bovine neutrophils. J. Dairy Sci. **94**, 152-164.

Stouthamer, R., Luck, R.F., Hamilton, W.D., 1990. Antibiotics cause parthenogenetic *Trichogramma* (Hymenoptera/Trichogrammatidae) to revert to sex. Proc. Natl. Acad. Sci. U. S. A **87**, 2424-2427.

Striebel, H.P., 1988. Proposed form to evaluate some histological aspects of macrofilarial morphology, its age dependent alterations and drug related changes in nodules of *Onchocerca volvulus* and *O. gibsoni*. Trop. Med. Parasitol. **39** Suppl 4, 367-389.

Strote, G., Brattig, N.W., Tischendorf, F.W., 1990. Ultrastructural study of the interaction between eosinophilic granulocytes and third and fourth stage larvae of *Onchocerca volvulus*. Acta Trop. **48**, 1-8.

Strubing, U., Lucius, R., Hoerauf, A., Pfarr, K.M., 2010. Mitochondrial genes for heme-dependent respiratory chain complexes are up-regulated after depletion of *Wolbachia* from filarial nematodes. Int. J. Parasitol. **40**, 1193-1202.

Suba, N., Shiny, C., Taylor, M.J., Narayanan, R.B., 2007. *Brugia malayi Wolbachia* hsp60 IgG antibody and isotype reactivity in different clinical groups infected or exposed to human bancroftian lymphatic filariasis. Exp. Parasitol. **116**, 291-295.

Subrahmanyam, D,. 1987. Antifilarials and their mode of action. "Filariasis", Ciba Foundation Symposium., John Wiley and Sons Ltd. **127**, 246-259.

Taguchi, M., Sampath, D., Koga, T., Castro, M., Look, D.C., Nakajima, S., Holtzman, M.J., 1998. Patterns for RANTES secretion and intercellular adhesion molecule 1 expression mediate transepithelial T cell traffic based ion analyses in vitro and in vivo. Journal of Experimental Medicine **187**, 1927-1940.

Tang, L., Ou, X., Henkleduhrsen, K., Selkirk, M.E., 1994. Extracellular and Cytoplasmic Cuzn Superoxide Dismutases from *Brugia* Lymphatic Filarial Nematode Parasites. Infection and Immunity **62**, 961-967.

Taylor, M.D., Harris, A., Nair, M.G., Maizels, R.M., Allen, J.E., 2006. F4/80+ alternatively activated macrophages control CD4+ T cell hyporesponsiveness at sites peripheral to filarial infection. J. Immunol. **176**, 6918-6927.

Taylor, M.D., LeGoff, L., Harris, A., Malone, E., Allen, J.E., Maizels, R.M., 2005a. Removal of regulatory T cell activity reverses hyporesponsiveness and leads to filarial parasite clearance in vivo. J. Immunol. **174**, 4924-4933.

Taylor, M.J., Bandi, C., Hoerauf, A., 2005c. *Wolbachia* bacterial endosymbionts of filarial nematodes. Adv. Parasitol. **60**, 245-284.

Taylor, M.J., Cross, H.F., Ford, L., Makunde, W.H., Prasad, G.B., Bilo, K., 2001. *Wolbachia* bacteria in filarial immunity and disease. Parasite Immunol. **23**, 401-409.

Taylor, M.J., Hoerauf, A., 1999. *Wolbachia* bacteria of filarial nematodes. Parasitol. Today **15**, 437-442.

Taylor, M.J., Makunde, W.H., McGarry, H.F., Turner, J.D., Mand, S., Hoerauf, A., 2005d. Macrofilaricidal activity after doxycycline treatment of *Wuchereria bancrofti*: a double-blind, randomised placebo-controlled trial. Lancet **365**, 2116-2121.

Tchakoute, V.L., Graham, S.P., Jensen, S.A., Makepeace, B.L., Nfon, C.K., Njongmeta, L.M., Lustigman, S., Enyong, P.A., Tanya, V.N., Bianco, A.E., Trees, A.J., 2006. In a bovine model of onchocerciasis, protective immunity exists naturally, is absent in drug-cured hosts, and is induced by vaccination. Proc. Natl. Acad. Sci. U. S. A **103**, 5971-5976.

Teixeira, L., Ferreira, A., Ashburner, M., 2008. The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in Drosophila melanogaster. PLoS Biol. **6**, e2.

Thomas, H.W., Mcgill, C.M., 1905. Some experiments in the treatment of trypanosomiasis. British Medical Journal **1905**, 1140-1143.

Thorne, K.J., Oliver, R.C., Barrett, A.J., 1976. Lysis and killing of bacteria by lysosomal proteinases. Infect. Immun. **14**, 555-563.

Tielsch, J.M., Beeche, A., 2004. Impact of ivermectin on illness and disability associated with onchocerciasis. Trop. Med. Int. Health **9**, A45-A56.

Timmermans, M.J.T.N., Ellers, J., 2009. *Wolbachia* endosymbiont is essential for egg hatching in a parthenogenetic arthropod. Evolutionary Ecology **23**, 931-942.

Tjabringa, G.S., Ninaber, D.K., Drijfhout, J.W., Rabe, K.F., Hiemstra, P.S., 2006. Human cathelicidin LL-37 is a chemoattractant for eosinophils and neutrophils that acts via formyl-peptide receptors. Int. Arch. Allergy Immunol. **140**, 103-112.

Townson, S., Tagboto, S., McGarry, H.F., Egerton, G.L., Taylor, M.J., 2006. *Onchocerca* parasites and *Wolbachia* endosymbionts: evaluation of a spectrum of antibiotic types for activity against *Onchocerca gutturosa* in vitro. Filaria. J. **5**, 4.

Trees, A.J., Graham, S.P., Renz, A., Bianco, A.E., Tanya, V., 2000. *Onchocerca ochengi* infections in cattle as a model for human onchocerciasis: recent developments. Parasitology **120** Suppl, S133-S142.

Turner, J.D., Langley, R.S., Johnston, K.L., Egerton, G., Wanji, S., Taylor, M.J., 2006. *Wolbachia* endosymbiotic bacteria of *Brugia malayi* mediate macrophage tolerance to TLR- and CD40-specific stimuli in a MyD88/TLR2-dependent manner. J. Immunol. **177**, 1240-1249.

Turner, J.D., Langley, R.S., Johnston, K.L., Gentil, K., Ford, L., Wu, B., Graham, M., Sharpley, F., Slatko, B., Pearlman, E., Taylor, M.J., 2009. *Wolbachia* lipoprotein stimulates innate and adaptive immunity through Toll-like receptors 2 and 6 to induce disease manifestations of filariasis. J. Biol. Chem. **284**, 22364-22378.

Tydell, C.C., Yount, N., Tran, D., Yuan, J., Selsted, M.E., 2002. Isolation, characterization, and antimicrobial properties of bovine oligosaccharide-binding protein. A microbicidal granule protein of eosinophils and neutrophils. J. Biol. Chem. **277**, 19658-19664.

Tydell, C.C., Yuan, J., Tran, P., Selsted, M.E., 2006. Bovine peptidoglycan recognition protein-S: antimicrobial activity, localization, secretion, and binding properties. J. Immunol. **176**, 1154-1162.

Underhill, D.M., Ozinsky, A., Hajjar, A.M., Stevens, A., Wilson, C.B., Bassetti, M., Aderem, A., 1999. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. Nature **401**, 811-815.

Vasselon, T., Detmers, P.A., Charron, D., Haziot, A., 2004. TLR2 recognizes a bacterial lipopeptide through direct binding. J. Immunol. **173**, 7401-7405.

Veldkamp, K.E., Heezius, H.C.J.M., Verhoef, J., van Strijp, J.A.G., van Kessel, K.P.M., 2000. Modulation of neutrophil chemokine receptors by *Staphylococcus aureus* supernate. Infection and Immunity **68**, 5908-5913.

Voegtlin, C., 1925. The pharmacology of arsphenamine (salvarsan) and related arsenicals. Physiological Reviews **5**, 63-94.

Vogel, B., Klinder, A., Sittig, D., Aust, G., 2005. Bovine eotaxin (CCL11)--an unusual member of the eotaxin group--attracts eosinophils in vitro but is not responsible for eosinophilia in the ovary. Vet. Immunol. Immunopathol. **107**, 67-77.

Volkmann, L., Fischer, K., Taylor, M., Hoerauf, A., 2003. Antibiotic therapy in murine filariasis (Litomosoides sigmodontis): comparative effects of

doxycycline and rifampicin on *Wolbachia* and filarial viability. Trop. Med. Int. Health **8**, 392-401.

Wahl, G., Ekale, D., Schmitz, A., 1998a. *Onchocerca ochengi*: assessment of the Simulium vectors in north Cameroon. Parasitology **116** (Pt 4), 327-336.

Wahl, G., Enyong, P., Ngosso, A., Schibel, J.M., Moyou, R., Tubbesing, H., Ekale, D., Renz, A., 1998b. *Onchocerca ochengi*: epidemiological evidence of cross-protection against *Onchocerca volvulus* in man. Parasitology **116** (Pt 4), 349-362.

Walz, A., Burgener, R., Car, B., Baggiolini, M., Kunkel, S.L., Strieter, R.M., 1991. Structure and neutrophil-activating properties of a novel inflammatory peptide (ENA-78) with homology to interleukin 8. J. Exp. Med. **174**, 1355-1362.

Walzer, T., Marcais, A., Saltel, F., Bella, C., Jurdic, P., Marvel, J., 2003. Cutting edge: Immediate RANTES secretion by resting memory CD8 T cells following antigenic stimulation. Journal of Immunology **170**, 1615-1619.

Wang, C.C., 1995. Molecular Mechanisms and Therapeutic Approaches to the Treatment of African Trypanosomiasis. Annual Review of Pharmacology and Toxicology **35**, 93-127.

Wanji, S., Tendongfor, N., Nji, T., Esum, M., Che, J.N., Nkwescheu, A., Alassa, F., Kamnang, G., Enyong, P.A., Taylor, M.J., Hoerauf, A., Taylor, D.W., 2009. Community-directed delivery of doxycycline for the treatment of onchocerciasis in areas of co-endemicity with loiasis in Cameroon. Parasit. Vectors. **2**, 39.

Wanji, S., Tendongfor, N., Vuong, P.N., Enyong, P., Bain, O., 2002. The migration and localization of *Loa loa* infective and fourth-stage larvae in normal and immunosuppressed rodents. Annals of Tropical Medicine and Parasitology **96**, 823-830.

Wasmoen, T.L., Bell, M.P., Loegering, D.A., Gleich, G.J., Prendergast, F.G., McKean, D.J., 1988. Biochemical and amino acid sequence analysis of human eosinophil granule major basic protein. J. Biol. Chem. **263**, 12559-12563.

Weeks, A.R., Turelli, M., Harcombe, W.R., Reynolds, K.T., Hoffmann, A.A., 2007. From parasite to mutualist: rapid evolution of *Wolbachia* in natural populations of *Drosophila*. PLoS. Biol. **5**, e114.

Werren, J.H., Baldo, L., Clark, M.E., 2008. *Wolbachia*: master manipulators of invertebrate biology. Nat. Rev. Microbiol. **6**, 741-751.

Whale, T.A., Griebel, P.J., 2009. A sheep in wolf's clothes: can neutrophils direct the immune response? Vet. J. **180**, 169-177.

WHO Technical Report Series., 1995. Onchocerciasis and its control. 852, 25-63

Wildblood, L.A., Kerr, K., Clark, D.A., Cameron, A., Turner, D.G., Jones, D.G., 2005. Production of eosinophil chemoattractant activity by ovine gastrointestinal nematodes. Vet. Immunol. Immunopathol. **107**, 57-65.

Wildenburg, G., Darge, K., Knab, J., Tischendorf, F.W., Bonow, I., Buttner, D.W., 1994. Lymph nodes of onchocerciasis patients after treatment with ivermectin: reaction of eosinophil granulocytes and their cationic granule proteins. Trop. Med. Parasitol. **45**, 87-96.

Wildenburg, G., Korten, S., Mainuka, P., Buttner, D.W., 1998. Ivermectin influence on the mast cell activity in nodules of onchocerciasis patients. Trop. Med. Int. Health **3**, 918-925.

Wildenburg, G., Kromer, M., Buttner, D.W., 1996. Dependence of eosinophil granulocyte infiltration into nodules on the presence of microfilariae producing *Onchocerca volvulus*. Parasitol. Res. **82**, 117-124.

Wildenburg, G., Plenge-Bonig, A., Renz, A., Fischer, P., Buttner, D.W., 1997. Distribution of mast cells and their correlation with inflammatory cells around *Onchocerca gutturosa*, *O. tarsicola*, *O. ochengi*, and *O. flexuosa*. Parasitol. Res. **83**, 109-120.

Willems, J., Joniau, M., Cinque, S., Van, D.J., 1989. Human granulocyte chemotactic peptide (IL-8) as a specific neutrophil degranulator: comparison with other monokines. Immunology **67**, 540-542.

Woldehiwet, Z., Scaife, H., Hart, C.A., Edwards, S.W., 2003. Purification of ovine neutrophils and eosinophils: *Anaplasma phagocytophilum* affects neutrophil density. J. Comp Pathol. **128**, 277-282.

Wolstenholme, A.J., Rogers, A.T., 2005. Glutamate-gated chloride channels and the mode of action of the avermectin/milbemycin anthelmintics. Parasitology **131**, S85-S95.

Wong, D.T.W., Donoff, R.B., Yang, J., Song, B.Z., Matossian, K., Nagura, N., Elovic, A., Mcbride, J., Gallagher, G., Todd, R., Chiang, T., Chou, L.S.S., Yung, C.M., Galli, S.J., Weller, P.F., 1993. Sequential Expression of Transforming Growth-Factor-Alpha and Factor-Beta(1) by Eosinophils During Cutaneous Wound-Healing in the Hamster. American Journal of Pathology **143**, 130-142.

Worku, M., Morris, A., 2009. Binding of different forms of lipopolysaccharide and gene expression in bovine blood neutrophils. J. Dairy Sci. **92**, 3185-3193.

Wu, W., Chen, Y., Hazen, S.L., 1999. Eosinophil peroxidase nitrates protein tyrosyl residues. Implications for oxidative damage by nitrating intermediates in eosinophilic inflammatory disorders. J. Biol. Chem. **274**, 25933-25944.

Xie, H., Bain, O., Williams, S.A., 1994. Molecular phylogenetic studies on filarial parasites based on 5S ribosomal spacer sequences. Parasite **1**, 141-151.

Yadav, R., Larbi, K.Y., Young, R.E., Nourshargh, S., 2003. Migration of leukocytes through the vessel wall and beyond. Thromb. Haemost. **90**, 598-606.

Yanagisawa, S., Koarai, A., Sugiura, H., Ichikawa, T., Kanda, M., Tanaka, R., Akamatsu, K., Hirano, T., Matsunaga, K., Minakata, Y., Ichinose, M., 2009. Oxidative stress augments toll-like receptor 8 mediated neutrophilic responses in healthy subjects. Respir. Res. **10**, 50.

Yeruham, I., Perl, S., Sharony, D., Vishinisky, Y., 2002. Doxycycline toxicity in calves in two feedlots. J. Vet. Med. B Infect. Dis. Vet. Public Health **49**, 406-408.

Yu, C., Shi, Z.R., Chu, C.Y., Lee, K.H., Zhao, X., Lee, J.W., 2010. Expression of bovine granulocyte chemotactic protein-2 (GCP-2) in neutrophils and a mammary epithelial cell line (MAC-T) in response to various bacterial cell wall components. Vet. J. **186**, 89-95.

Zabucchi, G., Menegazzi, R., Cramer, R., Nardon, E., Patriarca, P., 1990. Mutual influence between eosinophil peroxidase (EPO) and neutrophils: neutrophils reversibly inhibit EPO enzymatic activity and EPO increases neutrophil adhesiveness. Immunology **69**, 580-587.

Zanetti, M., Litteri, L., Gennaro, R., Horstmann, H., Romeo, D., 1990. Bactenecins, defense polypeptides of bovine neutrophils, are generated from precursor molecules stored in the large granules. J. Cell Biol. **111**, 1363-1371.

Zhao, X., Lacasse, P., 2008. Mammary tissue damage during bovine mastitis: causes and control. J. Anim Sci. **86**, 57-65.

Zigmond, S.H., 1977. Ability of polymorphonuclear leukocytes to orient in gradients of chemotactic factors. J. Cell Biol. **75**, 606-616.

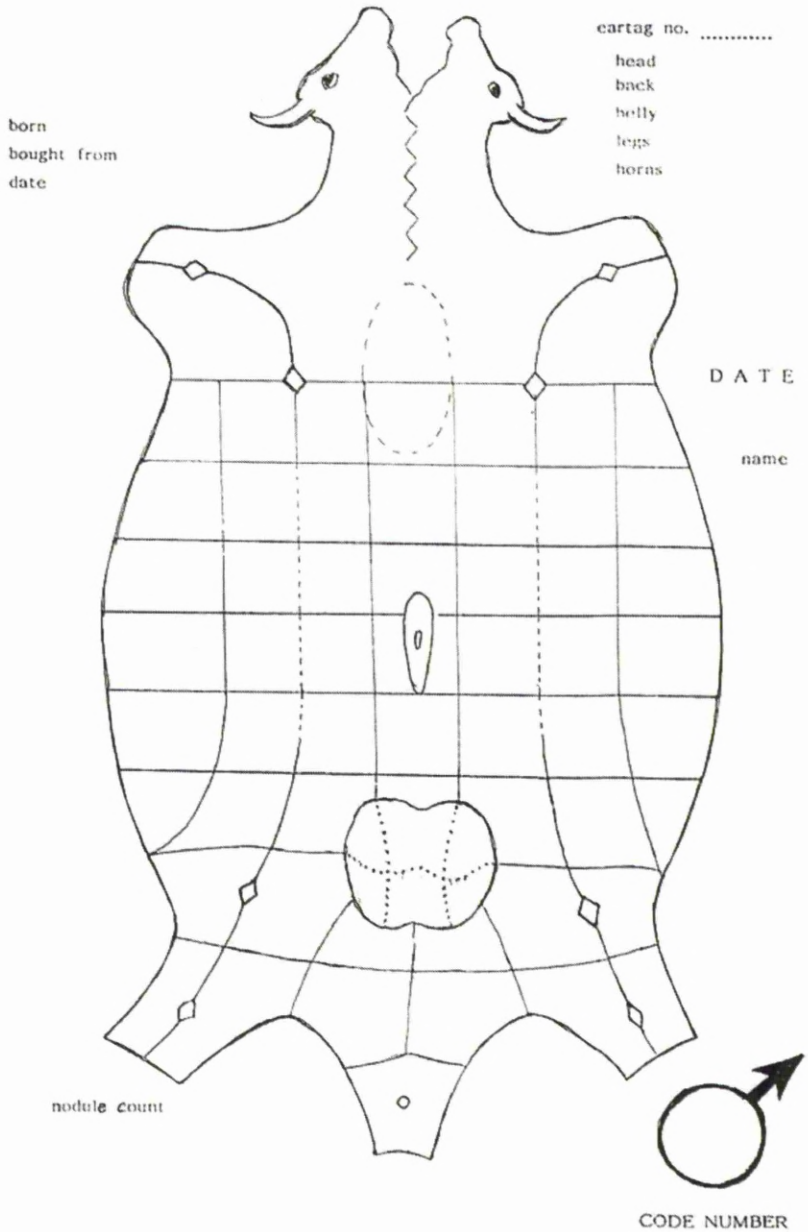
Zigmond, S.H., Hirsch, J.G., 1973. Leukocyte locomotion and chemotaxis. New methods for evaluation, and demonstration of a cell-derived chemotactic factor. J. Exp. Med. **137**, 387-410.

Zimin, A.V., Delcher, A.L., Florea, L., Kelley, D.R., Schatz, M.C., Puiu, D., Hanrahan, F., Pertea, G., Van Tassell, C.P., Sonstegard, T.S., Marcais, G., Roberts, M., Subramanian, P., Yorke, J.A., Salzberg, S.L., 2009. A whole-genome assembly of the domestic cow, Bos taurus. Genome Biology **10** (4), r42.

Zlotnik, A., Yoshie, O., 2000. Chemokines: a new classification system and their role in immunity. Immunity **12**, 121-127.

12. Appendices

12.1. The 'hide map' used in the field to record locations of tattooed nodules.



12.2. Cattle receiving Terramycin® for recovery of *Wolbachia*-depleted *O. ochengi*.

Cow I.D	Nodule count (palpable)	Weigh t (kg)	Volume Terramycin ® I/V 200 mg ml^{-1} (ml) SID	Treatment start date	Treatment end date	Slaughter date	Nodules recovered
3	55	305	15.3	13.08.07	26.08.07	2.11.07	97
4	28	335	16.8	13.08.07	26.08.07	23.10.07	28
9	62	259	13.0	13.08.07	26.08.07	26.10.07	179
21	74	274	13.7	13.08.07	26.08.07	24.10.07	163
25	53	299	15.0	13.08.07	26.08.07	26.10.07	150
28	51	322	16.1	13.08.07	26.08.07	24.10.07	49
31	112	225	11.3	13.08.07	26.08.07	3.11.07	75
32	113	297	14.9	13.08.07	26.08.07	25.10.07	216
36	51	234	11.7	13.08.07	26.08.07	30.10.07	54
37	121	292	14.6	13.08.07	26.08.07	1.11.07	107
38	72	306	15.3	13.08.07	26.08.07	31.10.07	82
40	42	220	11.0	13.08.07	26.08.07	23.10.07	32
42	43	290	14.5	13.08.07	26.08.07	25.10.07	136
45	75	182	9.1	13.08.07	26.08.07	27.10.07	84
46	100	286	14.3	13.08.07	26.08.07	29.10.07	120

12.3. Schedule of excision of randomised nodule pairs

Animal	39	43	62	63	59	66	53	51	58	50	52	48	55	47	56	41	49	65
TP 0	1	1	1	4*	1*	9*	5	9	5	5*	9*	4*	7*	5*	10*	7	5	1
2	10	2	4	6	9	1	7	2	9	4	1	9	4	4	5	10	3	7
4	9	7	5	1*	10*	4*	1	8	2	3*	4*	1*	2*	3*	2*	2	6	3
8	2	6	7	9	3	7	3	4	10	2	2	10	3	2	3	5	7	4
12	3	5	9	7*	4*	3*	2	1	6	1*	5*	8*	10*	7*	6*	3	8	6
24	5	4	3	5	8	6	4	3	3	10	3	5	5	10	4	8	10	5
36	7	3	10	10*	7*	8*	8	5	1	8*	8*	3*	8*	8*	7*	4	2	10
48	6	10	8	3	5	10	10	10	4	7	10	2	1	1	8	9	4	8
54	4	9	6	2*	2*	5*	6	7	8	9*	6*	6*	9*	6*	1*	1	9	2

***One extra nodule removed for EM.**

12.4. Nodulectomies:

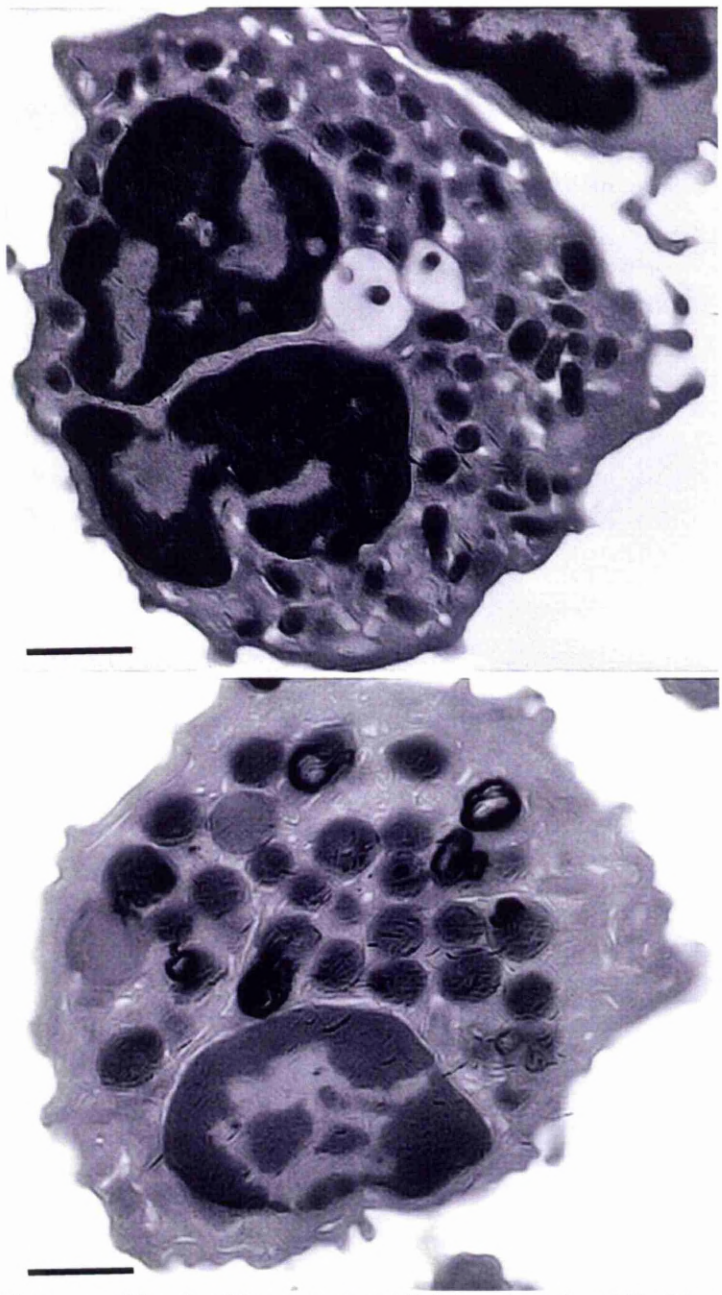
Upper photographs shows a Gudali cow in left lateral recumbency, wound being sutured following nodulectomy. Lower photograph shows a skin incision around a nodule pair.

12.5. Table used to record parameters identified in IHC sections (continued on next page)

Slide code	Cow#	TP	Tx	No of Degranulated eos regions	Area no.	# degran eos per unit area			Zone 0	Zone 1	Zone 2
								Cells D.E E N GC M PC L			
Key: Cells: cells found in this zone Scored in comparison to each other DE: degranulating eosinophils E: eosinophils N: neutrophils GC: giant cells M: macrophages PC: plasma cells L: lymphocytes SH material: Y or N Sex of worm: M/F/U Cuticle integrity: S=smooth; R=rough;B=broken, U= unknown Hypodermis integrity: Y= thin, uniform, nuclei seen; N=vacuolated, unhealthy-looking Hypodermis precipitate (ppt) score + to +++ Genital tract integrity: Y= smooth wall, normal looking; N=wall damaged, unhealthy looking Genital tract ppt score: + to+++ Genital tract presence of mff: Y= mff present;D=damaged; N=no mff present							Worm sections	SH material? Sex			
								Cuticle integrity			
								Hypodermis ppt score			
								Hypodermis integrity			
								Genital tract integrity			
								Genital tract ppt score			
								Genital tract presence of mff			
								Mff ppt?			
								Parasite status			
					Area no.	# degran eos per unit area			Zone 0	Zone 1	Zone 2
								Cells D.E E N GC M PC L			
							Worm	SH material Sex			
								Cuticle integrity			
								Hypodermis ppt score			

Mff ppt: + to +++ **Parasite status: H= healthy; P= productive: N= non-productive; D=dead; U=unsure**				**Hypodermis integrity**			
				Genital tract integrity			
				Genital tract ppt score			
				Genital tract presence of mff			
				Mff ppt?			
				Parasite status			

12.6. Ultrastructure of bovine granulocytes.



TEM images of bovine blood neutrophil (a) and eosinophil. (b). Scale bars = 1 μm

dependent variable (copy no.)	day	fixed factor	*F*	d.f.	*P*
16S rRNA	3	drug	27.886	1, 21	**< 0.001**
		concentration	1.278	2, 21	0.299
		interaction	0.120	2, 21	0.887
	7	drug	13.984	1, 21	**0.001**
		concentration	2.092	2, 21	0.148
		interaction	1.005	2, 21	0.383
18S rRNA	3	drug	1.844	1, 21	0.189
		concentration	0.009	2, 21	0.991
		interaction	0.244	2, 21	0.786
	7	drug	27.335	1, 21	**< 0.001**
		concentration	1.721	2, 21	0.203
		interaction	0.960	2, 21	0.399
16S:18S rRNA ratio	3	drug	36.675	1, 21	**< 0.001**
		concentration	0.761	2, 21	0.480
		interaction	0.070	2, 21	0.933
	7	drug	112.421	1, 21	**< 0.001**
		concentration	0.089	2, 21	0.916
		interaction	0.104	2, 21	0.902

12.7. Results of the general linear models

12.8. Formulary

12.8.1. Chapter 3.1

Cryopreservation medium	32.5 ml Schneider's *Drosophila* medium (PromoCell) 32.5 ml Mitsuhashi/Maramorosh insect medium (PromoCell) 20 ml foetal calf serum 6 ml [200mM] L-glutamine 10 ml dimethyl sulfoxide
Growth medium (50 ml)	18.5 ml Schneider's *Drosophila* medium (PromoCell) 18.5 ml Mitsuhashi/Maramorosh insect medium (PromoCell) 10 ml foetal calf serum 3 ml L-glutamine
Maintenance medium (100 ml)	42 ml Schneiders *Drosophila* medium (Sigma) 42 ml Mitsuhashi/Maramorosh insect medium (PromoCell) 10 ml foetal calf serum (Sigma) 6 ml L-glutamine (Sigma)

12.8.2. Chapter 3.2

BSA/sucrose blocking buffer (100ml)	1g bovine serum albumin (Sigma) 5g sucrose (Sigma) ➢ Dissolve in wash buffer to a total volume of 100 ml.
Wash buffer (1 L)	➢ Dissolve 1.21 g Tris in 800 ml distilled water.

	➢ Adjust pH to 8.5 using 1M HCl ➢ Add 8.7 g NaCl ➢ Add 1 ml Tween ➢ Add distilled water to a final volume of 1 L
Sodium citrate buffer (1 L)	➢ Add 29.4 g sodium citrate powder to 900 ml distilled water. ➢ Adjust pH to 6.0 using 1 M HCl ➢ Add distilled water to a final volume of 1 L

12.8.3. **Chapter 3.3**

Ammonium chloride solution (1 L)	➢ Dissolve 7.38 g ammonium chloride in I L purified water and remove 42 ml ➢ Prepare 50 ml 0.5 M Tris (3.03 g Tris dissolved in purified water to make a final volume of 50 ml), and add 42 ml to the ammonium chloride solution ➢ Adjust to pH 7.4 using 1 M HCl ➢ Refrigerate at 4° C
Percoll® stock solution (20 ml)	➢ Add 2 ml of 10× PBS (Sigma) to 18 ml Percoll®
0.11 M sodium citrate buffer (1 L)	➢ Dissolve 32.35 g sodium citrate powder in 968 ml sterile water ➢ Adjust pH to 7.4 using 1 M HCl
Enriched DMEM (100 ml)	➢ Add 1 ml L-leucine (Sigma) and 2 ml L-glutamine to 100 ml DMEM ➢ Mix and remove 2.5 ml. ➢ Add 2.5 ml 10 × HEPES buffer ➢ Replace 1 ml DMEM with heat-treated serum
Polyacrylamide stacking gel	➢ 375 µl 30 % acrylamide ➢ 625 µl stacking gel buffer ➢ 1500 µl double distilled water

	➢ 12.5 µl 10% ammonium persulphate and 3.75 µl N,N,N',N'-tetramethylethane-1,2-diamine added immediately prior to pouring gel.
Polyacrylamide resolving gel	➢ 4.21 ml 30 % acrylamide ➢ 2.5 ml resolving gel buffer ➢ 3.34 ml double distilled water ➢ 37.5 µl 10% ammonium persulphate and 10 µl N,N,N',N'-tetramethylethane-1,2-diamine added immediately prior to pouring gel
1× Laemmli sample buffer	➢ 4 ml 1.5 M Tris-Cl pH 6.8 ➢ 10 ml glycerol ➢ 5 ml β-mercaptoethanol ➢ 2 g sodium dodecyl sulphate ➢ 1 ml 1% bromophenol blue
Colloidal Coomassie Brilliant Blue (1 L)	➢ 2.5 g Coomassie blue ➢ 500 ml methanol ➢ 100 ml acetic Acid ➢ Add double distilled water to a final concentration of 1 L
10 ×TGS buffer (1 L)	➢ 29 g Tris base ➢ 144 g Glycine ➢ 10 g sodium dodecyl sulphate ➢ Dissolve in double distilled water to a final volume of 1 L.
10 × Transfer buffer (1 L)	➢ 30.3 g Tris base ➢ 144 g glycine ➢ Dissolve in double distilled water to a final volume of 1 L ➢ Adjust pH to 8.3 using HCl
Blocking buffer (100 ml)	➢ 1 g bovine serum albumin ➢ 5 g sucrose ➢ Dissolve in wash buffer to a final volume of 100 ml

Wash buffer (1 L)	➢ Dissolve 1.21 g Tris in 800 ml double distilled water ➢ Adjust pH to 8.5 using 1 M HCl ➢ Add 8.7 g NaCl ➢ Add 1 ml Tween ➢ Add double distilled water to a final volume of 1 L

12.9. Supporting material: "A worm's best friend: recruitment of neutrophils by *Wolbachia* confounds eosinophil degranulation against the filarial nematode *Onchocerca ochengi*"

PROCEEDINGS OF THE ROYAL SOCIETY B

FirstCite e-publishing

Proc. R. Soc. B
doi:10.1098/rspb.2010.2367
Published online

A worm's best friend: recruitment of neutrophils by *Wolbachia* confounds eosinophil degranulation against the filarial nematode *Onchocerca ochengi*

Rowena D. E. Hansen[1], Alexander J. Trees[1], Germanus S. Bah[2], Udo Hetzel[1], Coralie Martin[3], Odile Bain[3], Vincent N. Tanya[2] and Benjamin L. Makepeace[1,*]

[1]*Liverpool School of Tropical Medicine, School of Veterinary Science and Institute of Infection and Global Health, University of Liverpool, Liverpool L69 7ZJ, UK*
[2]*Institut de Recherche Agricole pour le Développement, Regional Centre of Wakwa, Ngaoundéré, BP 65 Adamawa Region, Cameroon*
[3]*USM 307 Parasitologie Comparée et Modèles Expérimentaux, Muséum National d'Histoire Naturelle, 61 Rue Buffon, 75231 Paris Cedex 05, France*

Onchocerca ochengi, a filarial parasite of cattle, represents the closest relative of the human pathogen, *Onchocerca volvulus*. Both species harbour *Wolbachia* endosymbionts and are remarkable in that adult female worms remain viable but sessile for many years while surrounded by host cells and antibodies. The basis of the symbiosis between filariae and *Wolbachia* is thought to be metabolic, although a role for *Wolbachia* in immune evasion has received little attention. Neutrophils are attracted to *Wolbachia*, but following antibiotic chemotherapy they are replaced by eosinophils that degranulate on the worm cuticle. However, it is unclear whether the eosinophils are involved in parasite killing or if they are attracted secondarily to dying worms. In this study, cattle infected with *Onchocerca ochengi* received adulticidal regimens of oxytetracycline or melarsomine. In contrast to oxytetracycline, melarsomine did not directly affect *Wolbachia* viability. Eosinophil degranulation increased significantly only in the oxytetracycline group; whereas nodular gene expression of bovine neutrophilic chemokines was lowest in this group. Moreover, intense eosinophil degranulation was initially associated with worm vitality, not degeneration. Taken together, these data offer strong support for the hypothesis that *Wolbachia* confers longevity on *O. ochengi* through a defensive mutualism, which diverts a potentially lethal effector cell response.

Keywords: onchocerciasis; eosinophilia; filariasis; innate immunity; interleukin-8; GRO

1. INTRODUCTION

The maternally transmitted α-proteobacterium, *Wolbachia*, infects an estimated two-thirds of all arthropod species [1], but it also has a more limited distribution in certain parasitic nematodes (the family Onchocercidae, superfamily Filarioidea, order Spirurida [2,3]; and the family Pratylenchidae, order Tylenchida [4]). In arthropod hosts, it expresses a diverse array of phenotypes from reproductive alterations including cytoplasmic incompatibility, male killing and feminisation [5], to mutualistic roles such as metabolic provisioning and protection against pathogens [6–8].

Filarial nematodes harbouring *Wolbachia* cause two major neglected tropical diseases, lymphatic filariasis (elephantiasis) and onchocerciasis (River Blindness), which are responsible for a combined global morbidity of greater than 6 million disability-adjusted life-years [9]. The obligate dependency of *Wolbachia*-positive filariae on their symbiont was demonstrated by prolonged antibiotic treatment of filarial infections in animal models, which led to stunting and sterilization [10] or death of adult worms [11]. These findings have been successfully translated into a treatment for individual patients suffering from filarial diseases [12]. Additionally, *Wolbachia* contributes to the clinical presentation of filarial infections, since bacterial products released from both living [13] and dead [14] worms activate the mammalian innate immune system, triggering the release of pro-inflammatory mediators.

The complete genome sequence of the *Wolbachia* strain (*w*Bm) from the human lymphatic filariid *Brugia malayi* revealed that the bacterium carries the genes required to synthesize riboflavin and haem, while its filarial host does not [15]. These data reinforced the prevailing perspective, initiated by the antibiotic chemotherapy experiments, that *Wolbachia* in filariae are 'obligate mutualists', in contrast with their close cousins in arthropods, which could be broadly described as 'reproductive parasites' [16].

In filarial nematodes, the precise mechanism by which *Wolbachia* depletion leads to adult worm death has not

* Author for correspondence (blm1@liv.ac.uk).

Electronic supplementary material is available at http://dx.doi.org/10.1098/rspb.2010.2367 or via http://rspb.royalsocietypublishing.org.

Received 30 October 2010
Accepted 2 December 2010

been elucidated. In *Onchocerca ochengi*, a parasite of cattle that represents the closest relative of the human pathogen *Onchocerca volvulus* [17], prolonged, intermittent antibiotic regimens are adulticidal [11,18]; whereas short continuous treatment is not [19]. Moreover, antibiotic chemotherapy radically alters the nature of the inflammatory response around the adult worms [20]. These parasites reside in fibrous nodules (onchocercomata) and can live for a decade, surrounded by a host infiltrate that consists primarily of neutrophils, which are stimulated by *Wolbachia*-derived molecules [21,22]. Following endobacterial clearance by oxytetracycline, the neutrophil population gradually recedes and is replaced by an influx of eosinophils [20], the classic effector cell in helminth infections, which are conspicuous by their scarcity in untreated onchocercomata. Thus, it is possible that the neutrophilic response is not simply an inevitable consequence of the presence of an endobacterium within a parasite of mammals, but instead a sublime example of defensive mutualism, in which the fitness of the worm is increased by an admixture of immunological signals. We tested this hypothesis by comparing the local immune response to adult *O. ochengi* treated with either an antibiotic (oxytetracycline) or a conventional adulticide (melarsomine), since if the observed eosinophilia is merely a non-specific response to dead or moribund worms, it should also occur following melarsomine chemotherapy.

2. MATERIAL AND METHODS

(a) *Animals, field site and chemotherapy*

Ngaoundéré Gudali cattle (*Bos indicus*) naturally infected with *O. ochengi* (greater than or equal to 30 palpable intradermal nodules per animal) were purchased from markets across the Adamawa Region of Cameroon and assembled at the Institut de Recherche Agricole pour le Développement (IRAD), Regional Centre of Wakwa.

The position of 23 nodules per animal was marked by peripheral tattoo and recorded by digital video and on a hard-copy 'hide map'. Using a statistical calculator to generate random numbers, 15 cows were allocated to three groups of five through elimination by ear tag number, and one bull was assigned to each group (since sufficient female animals were not available). One group received oxytetracycline (OXY; Terramycin LA, Pfizer) at 10 mg kg^{-1} intravenously, daily for 14 days followed by 20 mg kg^{-1} intramuscularly, monthly for five months; a regimen that kills greater than 80 per cent of adult worms by 12 months after the start of treatment [19]. One group was treated with melarsomine (MEL; Cymelarsan, Merial) solution at 4 mg kg^{-1} intravenously, every other day for 3 days, which has been demonstrated to be 100 per cent adulticidal [23]. This drug kills the majority of worms by 20 weeks, although some nodules containing fragments of degenerated parasites may persist for several years after treatment [18]. The third group of animals served as untreated controls (CON). Nodules were excised under local anaesthesia at nine predetermined time-points, and the sequence of nodulectomies was randomized (as for group allocation) with regard to their position on the animal. At every time-point, one nodule per animal was fixed by injection with 10 per cent neutral-buffered formalin, and another was preserved by injection with RNA*later* (Sigma) and stored at −20°C. A third nodule was removed at every other time-point and fixed by injection with 5 per cent glutaraldehyde in 75 mM sodium cacodylate buffer, pH 7.4 and stored at 4°C.

(b) *Histopathology of the bovine cellular response*

Paraffin-embedded, formalin-fixed nodules were cut into 4 μm sections. For quantitative histopathological analysis (performed by an individual 'blinded' to treatment group), the sections were stained with Giemsa and digital images of the entire nodule at 20× magnification were obtained on an Eclipse 80i microscope (Nikon UK, Kingston-upon-Thames) using a DS-U1 camera control unit and NIS-Elements Basic Research 3.0 software. The diameter and area of each nodule were calculated; and granulocytes within a continuous transect of the nodule midsection were differentiated by eye at 200× [20], digitally tagged as neutrophils or eosinophils, and quantified within an overlaid grid. Counts were normalized to cells per square-millimetre. Degranulating eosinophils (DE) were semi-quantified on a four-point scale as number per worm section (0 = 0, 1 = <1, 2 = 1–10, 3 = >10) within each transect.

(c) *Spatial distribution of eosinophil degranulation in relation to worm integrity*

In all nodules from 8, 12 and 24 weeks post-treatment (wpt) containing DE, the spatial distribution of eosinophil clusters containing greater than 10 cells was analysed in relation to the presence, proximity and integrity of worm sections by light microscopy. Three concentric zones around each eosinophil cluster were defined as: 0 = <50 μm; 1 = >50, <100 μm; and 2 = > 100, <150 μm. The structural integrity and reproductive status of the worm sections were scored according to a simplified version of the form proposed by Striebel [24], using categories presented in table 1.

Wolbachia were localized in worm sections by immunohistochemistry using a polyclonal antibody against *Wolbachia* surface protein (kindly donated by M. Casiraghi, University of Milan), as previously described [19]. Glutaraldehyde-fixed nodules were prepared for electron microscopy as detailed previously [19], and micrographs were obtained on an H-7100 transmission electron microscope (Hitachi High Technologies, Krefeld, Germany) fitted with a charge-coupled device camera (Hamamatsu, Massy, France) and a digital image acquisition software (Advanced Microscopy Techniques, Danvers, MA, USA). Bovine neutrophils and eosinophils were differentiated according to ultrastructural characteristics (see the electronic supplementary material, appendix S1, for details).

(d) *Analysis of gene expression following drug treatment* in vitro *and* in vivo

Aedes albopictus mosquito cells naturally infected with *Wolbachia* (cell-line Aa23) were cultured in 25 cm^2 flasks as previously described [25]. The cells were treated in quadruplicate for 3 or 7 days without treatment, or with melarsomine dihydrochloride (generously donated by C. Marcato, Merial, Toulouse) or doxycycline hyclate (Fluka) at three concentrations. The highest concentration applied (0.25 μg ml^{-1}) represented the minimal bactericidal concentration for doxycycline against *Wolbachia* [26]. The viability of *Wolbachia* and host cell physiology was determined by quantitative reverse-transcriptase (qRT)-PCR, using assays for *Wolbachia* 16S rRNA and *A. albopictus* 18S rRNA as detailed elsewhere [25].

Table 1. Associations between presence and integrity of *O. ochengi* adult worm sections in bovine nodules and degranulating eosinophil (DE) score at eight weeks post-treatment. (Bold type indicates statistical significance. The associations between degranulation and worm integrity were positive in all cases; i.e. DE scores were moderate and/or high where the sections exhibited normal structure. Sample sizes varied between analyses because only worm sections that could be scored unambiguously for each characteristic were included. Zone boundaries were measured in micrometres.)

observation	scoring system	value of χ^2 or r_s	n	association with DE score, p
presence of worm	absent = 0; present = 1	9.319[a]	135	**0.020**
proximity to worm (if present)	zone 0: <50; zone 1: >50, <100; zone 2: >100, <150	−0.334[b]	120	**<0.001**
genital tract integrity	damaged wall = 0; smooth wall = 1 (normal)	14.745[a]	43	**<0.001**
cuticle integrity	broken = 0; rough = 1; smooth = 2 (normal)	0.228[b]	115	**0.014**
hypodermal integrity	vacuolated = 0; thin = 1 (normal)	9.993[a]	111	**0.011**
reproductive status	non-productive = 0; productive = 1 (normal)	7.301[a]	109	**0.026**
intrauterine microfilariae	none = 0; present, damaged = 1; present, normal = 2	0.089[b]	113	0.350

[a]Fisher's exact test.
[b]Spearman's ρ.

For gene expression analysis in nodules preserved in RNA*later*, the tissue was cut into pieces of 1–2 mm^3 and homogenized at IRAD in Lysis-Binding Solution from an RNAqueous-Midi Kit (Ambion), using a T10 basic Ultra-Turrax disperser (IKA, Staufen, Germany) with an 8 mm dispersing element. The homogenates were stored at −80°C and shipped to the UK on dry ice, where the remainder of the kit manufacturer's protocol was followed. Total RNA was further purified by lithium chloride precipitation, dissolved in 0.1 mM ethylene diamine tetra-acetic acid, and quantified on a Nanodrop-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). If necessary, the RNA was concentrated in a Nanosep Omega 30K centrifugal device (Pall Life Sciences); and 1 µg was reverse-transcribed and assayed for *Wolbachia*, *O. ochengi*, and bovine transcripts using SYBR Green I (SensiMix Two-Step Kit, Quantace) on a MiniOpticon Real-Time PCR Detection System (Bio-Rad, Hemel Hempstead). For further details of qRT-PCR assay design, see the electronic supplementary material, appendix S2.

(e) *Statistical analysis*

Two cows (one each in the CON and MEL groups) died from causes unrelated to this study before the experiment was completed, and data from these animals were excluded from the statistical analyses. After normalization by log transformation, independent gene expression data were analysed using univariate general linear model (GLM), whereas equivalent data from related samples were subjected to GLM repeated measures. The Friedman test (with exact significance) was applied to time-course data in the histopathological analyses. Since onchocercomata resolve after worm death, nodules could not be obtained from all pre-determined sites at later time-points. Therefore, data from 24 to 36 wpt, and 48 to 54 wpt, were collapsed into one category each in the Friedman analysis, incorporating mean values where more than one sample per animal was available. To test for associations or correlations between DE score and worm status, Fisher's exact test (for two categories) or Spearman's ρ (for three categories) was used, respectively. All analyses were performed in PASW Statistics 17.0 (SPSS Inc., Chicago, IL, USA), applying a critical probability of $p < 0.05$. For full details of GLM design and post hoc analyses, see the electronic supplementary material, appendix S3.

3. RESULTS

(a) *Effect of melarsomine on* Wolbachia *in vitro*

The results from treatment of the Aa23 cell-line demonstrated that conversely to doxycycline, melarsomine preferentially targets eukaryotic cells and it exhibits no inhibitory or bactericidal activity against *Wolbachia* (see the electronic supplementary material, appendix S4 for details).

(b) *Effects of treatments on nodule histopathology*

Quantitative analysis revealed striking differences in the nodular eosinophil : neutrophil ratio between treatment groups (figure 1*a*). In the CON group, neutrophils vastly outnumbered eosinophils (overall median ratio = 0.004) and there was no significant change in the composition of the granulocytic infiltrate over time (Friedman test: $\chi^2 = 5.6$, $p = 0.507$). A modest increase in the eosinophil : neutrophil ratio was observed in the MEL group, culminating in a shift towards eosinophilia in resolving nodules at the termination of the experiment, although this was not statistically significant (figure 1*a*; Friedman test: $\chi^2 = 6.0$, $p = 0.473$). However, there was a much larger and sustained expansion of the nodular eosinophil population in the OXY group, which peaked in a statistically significant, 275-fold increase in the median eosinophil : neutrophil ratio by 36 wpt (figure 1*a*; Friedman test: $\chi^2 = 19.3$, $p < 0.001$). Median nodule area gradually reduced during both adulticidal treatments (figure 2*b*) as the worms were eliminated and this was statistically significant in the MEL group (Friedman test: $\chi^2 = 11.1$, $p = 0.012$), although only a non-significant trend was apparent in the OXY group (Friedman test: $\chi^2 = 10.3$, $p = 0.096$). When DE adjacent to worm sections were analysed separately on a semi-quantitative scale, a clear distinction between the MEL and OXY treatment groups was observed (figure 1*c*). In the MEL group (in common with the CON group), the median

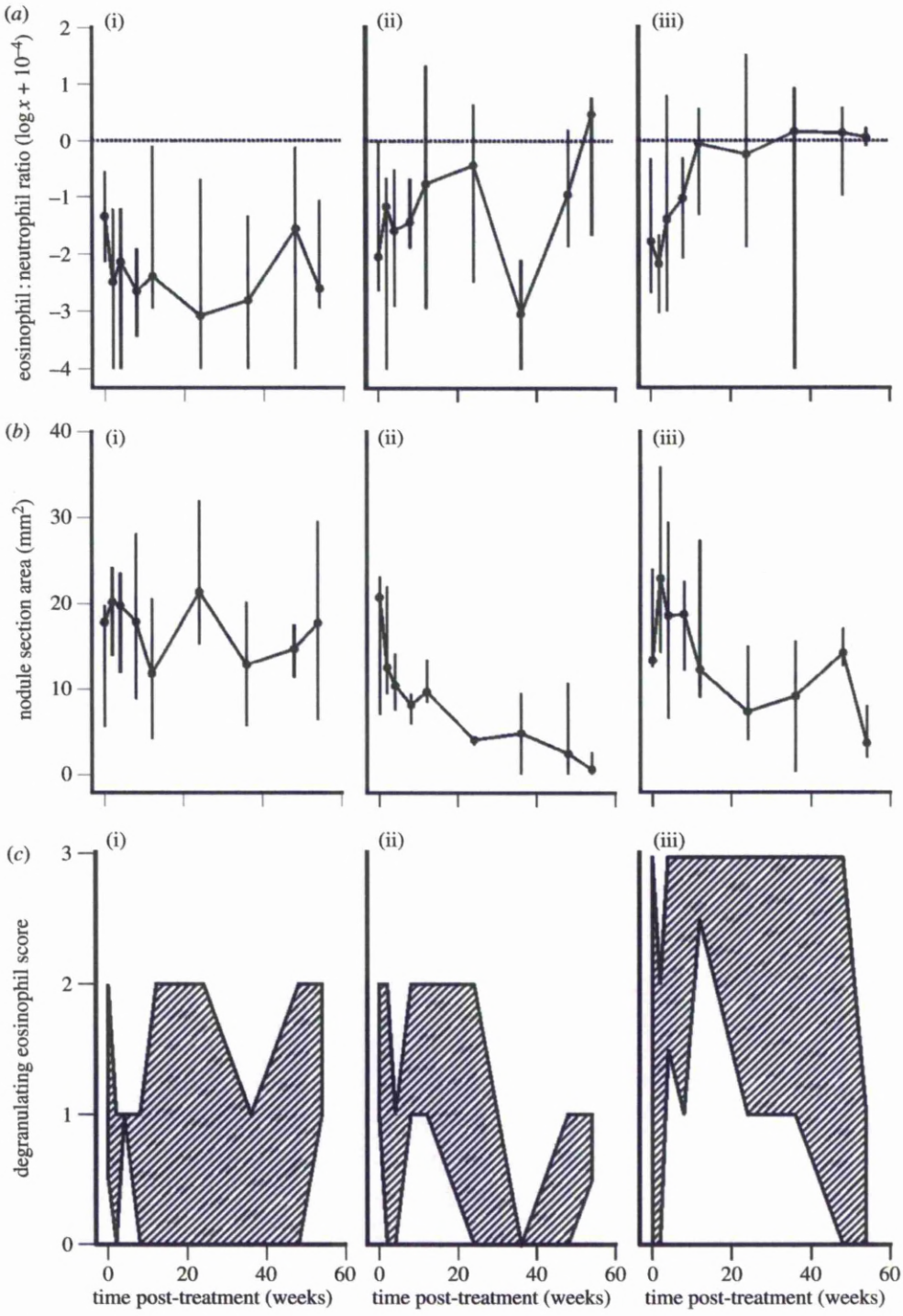


Figure 1. Bovine granulocyte responses in *O. ochengi* onchocercomata following (*a*(iii), *b*(iii), *c*(iii)) oxytetracycline ($n = 6$) or (*a*(ii), *b*(ii), *c*(ii)) melarsomine chemotherapy ($n = 5$), or (*a*(i), *b*(i), *c*(i)) in the absence of treatment ($n = 5$). (*a*) Eosinophil : neutrophil ratios (median and range). The horizontal dotted line represents parity between the two cell types. (*b*) Nodule section area (median and range). (*c*) Degranulating eosinophil scores per worm section (where 0 = 0, 1 = <1, 2 = 1–10, 3 = >10). The shaded area represents the region bounded by the median and maximum scores.

score did not exceed 1 throughout the experiment, and no significant change over time was detected (Friedman test, MEL: $\chi^2 = 4.9$, $p = 0.605$; CON: $\chi^2 = 3.9$, $p = 0.755$). By contrast, there was a marked, statistically significant peak in DE in the OXY group at 12 wpt (figure 1*c*; Friedman test: $\chi^2 = 11.9$, $p = 0.042$) and the maximum score of 3 (greater than 10 DE per worm section) was only recorded herein.

The spatial distribution of granulocytic infiltration was investigated during the period of maximum divergence in DE activity between treatment groups (12 wpt). Onchocercomata from CON animals exhibited neutrophilic infiltrates in close proximity to the cuticle of the adult worms (figure 2*a*), whereas eosinophils were extremely scarce and located near the outer capsule. Extensive Splendore-Hoeppli phenomena were common around

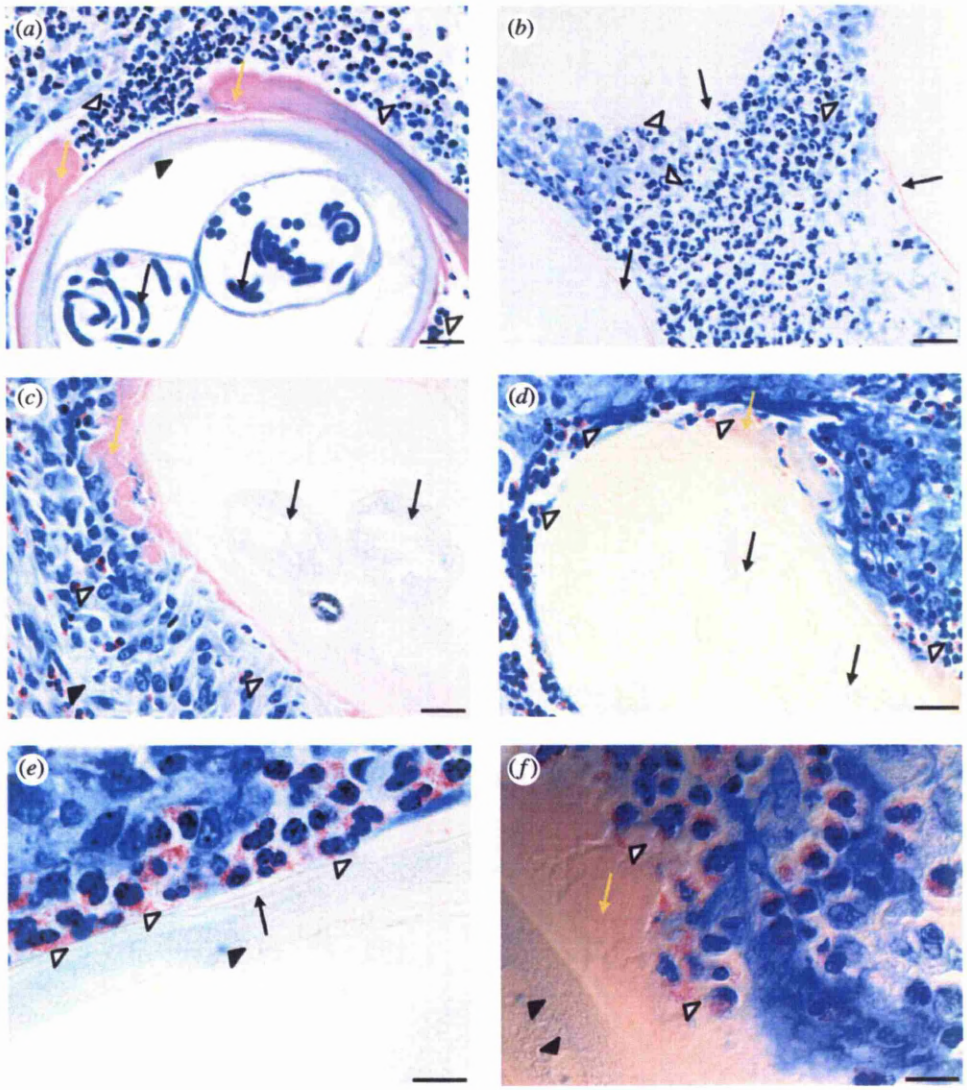


Figure 2. Histopathology of *O. ochengi* onchocercomata at 12 weeks. (*a*) Untreated onchocercoma with typical accumulations of neutrophils (open arrowheads) and Splendore-Hoeppli (SH) deposits (yellow arrows) on the worm cuticle. A cross section of an adult female worm shows the hypodermis (closed arrowhead) and microfilariae (black arrows) in the paired uteri. (*b*) A nodule from the melarsomine (MEL) group, displaying abundant neutrophils (open arrowheads) between the cuticles of three worm sections (black arrows). (*c*) A MEL-treated nodule with eosinophils (open arrowheads) and degranulating eosinophils (DE; closed arrowhead) some distance from a worm section. The worm has accumulated SH deposits (yellow arrow) and the uteri are empty (black arrows). (*d*) A nodule from the oxytetracycline (OXY) group shows numerous DE clusters (open arrowheads) around the cuticle, SH phenomenon (yellow arrow) and empty uteri (black arrows). (*e*) A higher power image from an OXY-treated nodule reveals DE and free granules (open arrowheads) in contact with the worm cuticle (black arrow) overlaying the hypodermis (closed arrowhead). (*f*) Phase-contrast image of a section from an OXY-treated nodule displaying eosinophil granules (open arrowheads) embedded in SH deposits (yellow arrow); filarial nuclei are also visible in the hypodermis (closed arrowheads). All sections were stained with Giemsa. Scale bars: (*a*—*d*) 20 μm; (*e*,*f*) 10 μm.

female worms (figure 2*a*). In the MEL group, neutrophils remained abundant between worm sections (figure 2*b*); although a modest increase in eosinophils was observed near to some female worms (figure 2*c*). However, eosinophils were very rarely in contact with the worm cuticle in the MEL group and degranulation was infrequent, usually occurring distally to worm sections (figure 2*c*). In marked contrast, eosinophils accumulated in layers around the worm cuticle in the OXY group, while neutrophils had become scarce (figure 2*d*). Furthermore, the majority of eosinophils surrounding the worms in the OXY group were undergoing degranulation (figure 2*d*). At higher magnification, clusters of eosinophil granules could be observed in direct contact with the epicuticle (figure 2*e*) or embedded within the Splendore-Hoeppli deposits adjacent to the cuticle (figure 2*f*).

(c) *Analysis of factors associated with eosinophil degranulation*

To investigate the factors that may trigger eosinophil degranulation, nodules that exhibited DE in the transect analysis at 8, 12 or 24 wpt were carefully inspected to locate all clusters of greater than 10 eosinophils *in toto*. One-hundred-and-forty such clusters were identified, 89 per cent of which were in OXY-treated onchocercomata.

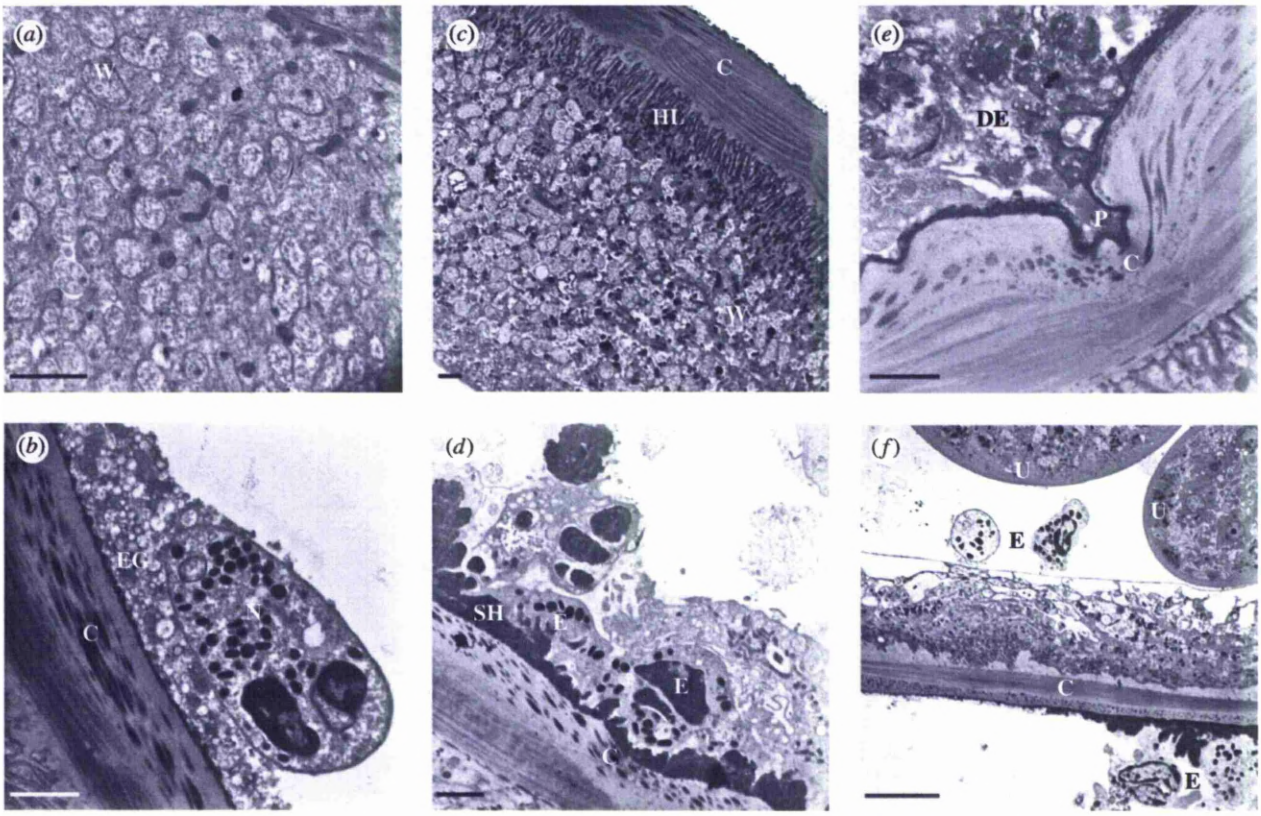


Figure 3. Ultrastructure of *O. ochengi* onchocercomata at 12 weeks. (*a*) Hypodermis of an untreated worm showing abundant *Wolbachia* endosymbionts (W). (*b*) The cuticle (C) of an untreated worm displaying normal structure, alongside an adherent neutrophil (N) surrounded by numerous extracellular granules (EG). (*c*) A worm section from the melarsomine (MEL) group displaying intact *Wolbachia* (W) beneath the hypodermal lamellae (HL) and the cuticle (C). (*d*) Eosinophils (E) attached to Splendore-Hoeppli deposits (SH) on the cuticle (C) of a worm from the MEL group. (*e*) The pseudopodium (P) of a degranulating eosinophil (DE) entering a cleft in the cuticle (C) of an oxytetracycline (OXY)-treated worm. (*f*) Two eosinophils (E) adjacent to the paired uteri (U) within the pseudocoelomic cavity of a worm from the OXY group; eosinophils are also visible on the host side of the cuticular (C) interface. Note the highly vacuolated hypodermis. Scale bars: (*a–e*) 2 μm; (*f*)10 μm.

At 8 wpt, the DE score for these clusters showed a weak but statistically significant negative correlation with neutrophil counts (Spearman's ρ: $r_s = -0.259$, $n = 135$, $p = 0.002$). At the same time-point, there was a statistically significant association (Fisher's exact test) between the DE score and the presence of worm sections in the microscopic field, and a significant positive correlation (Spearman's ρ) between the DE score and the proximity of worm sections (table 1). Moreover, there were statistically significant positive associations or correlations between the DE score and normal morphological features in the worm sections (smooth genital tract and cuticle, thin hypodermal cords and productive gonads), although not with intrauterine microfilariae *per se* (table 1). At 12 and 24 wpt, there were no significant associations between the DE score and these measures of morphological integrity. This was probably because less than 10 per cent of worm sections had a normal appearance at these time-points, producing extremely low expected frequencies in the analyses.

(d) *Effects of treatments on worm ultrastructure*

In worm sections from the CON group at 12 wpt, *Wolbachia* endobacteria were extremely abundant in the hypodermis (figure 3*a*). Neutrophils adjacent to the cuticle were frequently observed undergoing degranulation in the CON group, although this did not cause any discernible damage (figure 3*b*). In the MEL group, *Wolbachia* remained at high densities in the hypodermal cords (figure 3*c*) in worms that were not in the advanced stages of resorption. Eosinophils were occasionally seen close to the cuticle, but degranulation was very infrequent and the outer layers of the cuticle remained unmodified (figure 3*d*). By contrast, the ultrastructure of nodules in the OXY group at 12 wpt revealed unique alterations. Eosinophil degranulation in direct contact with the worm cuticle was common and associated with pronounced deformation of the outer cuticular layers (figure 3*e*). Furthermore, parasite integrity was clearly compromised, as eosinophils could be observed free within the body cavity of the worms (figure 3*f*).

(e) *Effects of treatments on gene expression in onchocercomata*

To determine the relative effect of MEL or OXY chemotherapy on the viability of *O. ochengi* and *Wolbachia* between 8 and 24 wpt, RNA transcripts of glutathione *S*-transferase 1a (*Oo*GST1a) were quantified from the former and 16S rRNA from the latter, and the ratio was calculated. Since nodules vary in worm content, the ratio between the expression levels of two genes is likely to be more informative than the absolute counts for

either transcript alone. Both treatments showed a non-significant trend towards reduction in expression of *Oo*GST1a over this time period, which approached the critical probability only in the MEL group (GLM repeated measures, Tamhane's T2 post hoc test, MEL versus CON: mean difference = −2.3 log, $p = 0.069$; see the electronic supplementary material, appendix S5, for data and additional details of the analysis). However, for 16S rRNA, both treatments induced a significant decrease in transcript counts relative to the CON group, although this was more substantial in the OXY group (see the electronic supplementary material, appendix S5; GLM repeated measures, Tukey's post hoc test, MEL versus CON: mean difference = −2.1 log, $p = 0.016$; OXY versus CON: mean difference = −3.1 log, $p = 0.001$). Importantly, the 16S rRNA : *Oo*GST1a ratio was significantly reduced relative to the CON group only in OXY-treated nodules (see the electronic supplementary material, appendix S5; Tamhane's T2 test: mean difference = −2.1 log, $p < 0.001$); and furthermore, this ratio was significantly lower in the OXY group than in the MEL group (Tamhane's T2 test: mean difference = −2.4 log, $p < 0.001$). These data were corroborated by immunohistochemistry for *Wolbachia* surface protein in worm sections from 12 wpt, which showed retention of *Wolbachia* in worms that had not yet disintegrated after the MEL treatment; whereas staining in worms from the OXY group was weak or negative (see the electronic supplementary material, appendix S6).

To assess local cellular immune responses to different treatments in the bovine host, transcripts for key cytokines involved in the activation and chemotaxis of neutrophils (interleukin-8 (IL-8) and growth-regulated (GRO) family) and eosinophils (IL-5 and eotaxin) were quantified between 8 and 24 wpt. In the OXY group, the IL-8 : 28S rRNA ratio was significantly lower than that observed in the CON group (figure 4*a*; GLM repeated measures, Tukey's post hoc test: mean difference = −1.0 log, $p = 0.010$; see the electronic supplementary material, appendix S4, for additional statistical details); whereas in the MEL group, IL-8 levels were not significantly different to those in CON nodules (Tukey's test: mean difference = −0.7 log, $p = 0.107$). Similarly, the GRO : 28S rRNA ratio was significantly reduced in the OXY group relative to CON levels (figure 4*b*; GLM repeated measures, Tukey's post hoc test: mean difference = −0.7 log, $p = 0.044$), but not between the MEL and CON groups (Tukey's post hoc test: mean difference = −0.4 log, $p = 0.296$). However, neither treatment had a significant effect on the expression of IL-5 (GLM repeated measures, Tukey's post hoc test, MEL versus CON: mean difference = 0.09 log, $p = 0.918$; OXY versus CON: mean difference = −0.03 log, $p = 0.991$; data not shown) or eotaxin genes (GLM repeated measures, Tukey's post hoc test, MEL versus CON: mean difference = −0.5 log, $p = 0.641$; OXY versus CON: mean difference = −0.3 log, $p = 0.814$; data not shown).

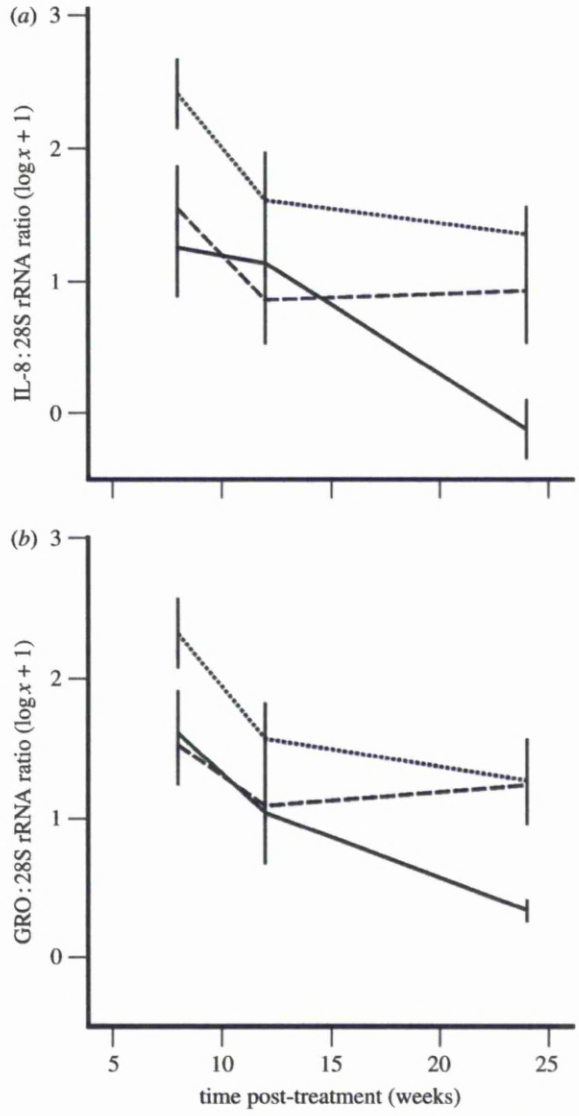


Figure 4. Quantification of bovine transcripts (normalized against 28S rRNA) for the neutrophilic chemokines (*a*) interleukin-8 (IL-8) and (*b*) the growth-regulated (GRO) protein family in onchocercomata treated with oxytetracycline ($n = 6$) or melarsomine ($n = 5$), or in the absence of treatment ($n = 5$). Lines represent the mean ± s.e. (*a*,*b*) Dotted line, control; dashed line, melarsomine; solid line, oxytetracycline.

4. DISCUSSION

A role for the immune system in the mechanism of action of antibiotics against adult *Onchocerca* spp. was first identified in a sequential study of cellular changes in *O. ochengi* onchocercomata treated with different regimens of oxytetracycline [20]. Eosinophils were implicated in worm killing because a relatively short, non-adulticidal antibiotic regimen caused a transient depletion of *Wolbachia* and a partial transition in the nodule towards eosinophilia. This was reversed when the endosymbionts recrudesced, leading to restoration of the neutrophil population and survival of the adult worms. However, without a concurrent comparison with non-antibiotic chemotherapy, the possibility that the observed eosinophilia was simply a secondary response to worm morbidity could not be excluded. In the present study, we rigorously evaluated the effects of an arsenical compound, melarsomine, on *Wolbachia* to confirm that it had no direct bacteriostatic or bactericidal activity and thus could serve as a control adulticide. Data obtained

rom *in vitro* treatment of the Aa23 cell-line demonstrated io deleterious effects of melarsomine on the endobacteria ınd this was corroborated by *ex vivo* analyses of *Wolbachia* n onchocercomata, where any reduction in endosym-)iont density was secondary to disruption of worm ntegrity.

Oxytetracycline treatment elicited a relatively early infil-ration of eosinophils and extensive degranulation on the vorms surface that (coupled with striking ultrastructural ibnormalities at 12 wpt) are strongly indicative of mmune-mediated damage. Importantly, during the initial vave of eosinophil degranulation at 8 wpt, these cells :xhibited their most intense activity against morphologi-:ally normal, reproductively active worms, with lower evels of degranulation observed against parasites that ılready showed pathological signs. This suggests that mor-bund worms do not trigger eosinophil degranulation in his system, but rather that the decrease in *Wolbachia* den-ity in viable worms is detected by the mammalian host, vhich responds with an intense but temporally restricted nflux of DE. By 12 wpt, this response had reached an ıcme, with the worms exhibiting damage that was almost :ertainly irreversible. However, the precise sequence of :vents leading to the penetration of eosinophils into the)seudocoelomic cavity was not clear, as we never observed :omplete rupture of the cuticle in OXY-treated worms at his time-point. It is perhaps more probable that following mmune-mediated alterations around apertures such as the ′ulva, the cells were able to diapedese internally.

Although one study has reported that doxycycline has ignificant anti-filarial activity against adult *Onchocerca gutturosa* worms cultured *in vitro* [27], these effects were)nly observed at a concentration of 100 times greater han the minimum bactericidal concentration against *Wolbachia* as determined in the Aa23 cell-line [26]; more-)ver, oxytetracycline had no detectable activity against *O. gutturosa in vitro* [27]. Thus, the immune system is)robably required for optimal activity of antibiotics ıgainst adult *Onchocerca* spp. By contrast, we found no :vidence for immunologically mediated damage following nelarsomine treatment, and the very late onset eosino-)hilia in resolving nodules and the limited degranulation)bserved in this treatment group are more consistent vith a role for these cells in clearance of worm fragments ınd in wound healing. Indeed, this process was also ıpparent in the latter stages following oxytetracycline che-notherapy (i.e. from 36 wpt), where eosinophil nfiltration, associated with much lower levels of degranu-ation, continued to increase in parallel with a trend owards reduced nodule size.

Three lines of evidence support the hypothesis that the *Wolbachia*-induced neutrophilia in untreated onchocerco-nata protects adult worms from eosinophil legranulation. First, transcripts for bovine chemokines hat are known to recruit neutrophils were significantly lownregulated in the OXY group but not the MEL group, suggesting that in the absence of neutrophilic sig-ıals released directly or indirectly by *Wolbachia*, worms ıttract eosinophils by 'default'. Second, at the critical ime-point of 8 wpt, eosinophil degranulation was inver-:ely correlated with neutrophil counts. Third, as liscussed above, recrudescence of *Wolbachia* can reconsti-ute the neutrophilia and 'rescue' the worms during the :arly stages of eosinophil infiltration [20].

This manipulation of the local inflammatory response by *Wolbachia* represents a defensive mutualism that is related to, but distinct from, the protection against patho-gens that it confers in arthropod hosts such as *Drosophila melanogaster* [6,8]. Indeed, in filarial worms, the host of the symbiont is itself a pathogen, and the external threat from which it requires defence is the immune system of a third organism. Closer parallels in arthropod hosts have been recently identified in two insect pests of plants. The leaf-mining moth *Phyllonorycter blancardella*, which appears to use *Wolbachia* to manipulate cytokinin levels in the leaves of apple trees, maintains 'green islands' in otherwise senescent leaves that facilitate the nutrition of the larval stages [28]. Furthermore, larvae of the beetle *Diabrotica virgifera virgifera*, a root pest of maize, can downregulate immune defence genes in the host plant when infected with *Wolbachia*, but loses this capacity after antibiotic treatment [29].

Since filariae have several life cycle stages and dimorphic sexes that reside in different anatomical com-partments in both vertebrate and arthropod hosts, it is probable that the role of *Wolbachia* is modulated during the worm development, as has been observed in the mutualism between the entomopathogenic nematodes *Heterorhabditis* spp. and *Steinernema* spp. and their enter-obacterial symbionts [30]. Haem biosynthesis can be plausibly linked to the production of ecdysteroid-like hor-mones that may regulate filarial moulting and embryogenesis, providing a mechanism by which the anti-biotic therapy induces embryostasis and retarded larval development [15]. However, these processes fall short of adulticidal effects, and do not provide an explanation for the role (if any) of *Wolbachia* in adult male worms. The 'patchy' distribution of *Wolbachia* among filariae (some genera with almost all members infected; other genera with only one member infected [2]) strongly implies that *Wolbachia* may confer a relatively minor evolutionary advantage during the initial contact with a population of new hosts, such as additional nutrients during periods of resource stress [31], or an immune evasion strategy that complements the numerous mech-anisms already available to the worms. The perhaps unparalleled success of *Wolbachia* as a symbiont may hinge on its ability to foster dependency by driving the loss of related functions from the nuclear genome of the host.

In this respect, the only known member of the genus *Onchocerca* that lacks *Wolbachia*, *Onchocerca flexuosa*, pro-vides the exception that proves the rule. Partial sequencing of the nuclear genome of this species has demonstrated that it harboured an ancestral symbiont, as it contains over 100 *Wolbachia* DNA fragments [32]. Unlike *O. ochengi* and *O. volvulus*, which establish remark-ably persistent infections, *O. flexuosa* has a lifespan estimated at only 1 year [33], with eosinophils constitut-ing a prominent component [34] of the onchocercomata in its cervine host. Since *O. ochengi* and *O. volvulus* take almost a full year to develop patent infections, *O. flexuosa* appears to have adopted an alternative reproductive strat-egy following loss of *Wolbachia*, in which it may have accelerated its development to sexual maturity to avoid elimination by eosinophils. By contrast, the fact that adult female worms of *O. ochengi* and *O. volvulus* can reproduce continuously for a decade, while in an almost

entirely sessile state in immunocompetent hosts, contends as yet another fascinating example of evolutionary innovation in *Wolbachia*.

All procedures performed on cattle in Cameroon were equivalent to those previously authorized by a Home Office Project Licence (Animals (Scientific Procedures) Act 1986) for experimental infections of cattle in the UK, and classified under the severity limit of 'mild'.

We are very grateful for the expert technical assistance of David Ekale and Henrietta Ngangyung and to the herdsmen of IRAD, Cameroon. We thank Theo Kanellos (Pfizer Animal Health) for his keen interest in the project, Marion Pope for sample preparation preceding electron microscopy, and Frans Van Gool (Merial) for coordinating the kind donation of Cymelarsan. This work was supported by Pfizer Animal Health and the EC (contract no. INCO-CT-2006-032321). R.D.E.H. received a doctoral training award from the BBSRC.

REFERENCES

1 Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A. & Werren, J. H. 2008 How many species are infected with *Wolbachia*?—a statistical analysis of current data. *FEMS Microbiol. Lett.* **281**, 215–220. (doi:10.1111/j.1574-6968.2008.01110.x)

2 Bain, O., Casiraghi, M., Martin, C. & Uni, S. 2008 The nematoda Filarioidea: critical analysis linking molecular and traditional approaches. *Parasite* **15**, 342–348.

3 Bandi, C., Anderson, T. J., Genchi, C. & Blaxter, M. L. 1998 Phylogeny of *Wolbachia* in filarial nematodes. *Proc. R. Soc. Lond. B* **265**, 2407–2413. (doi:10.1098/rspb.1998.0591)

4 Haegeman, A., Vanholme, B., Jacob, J., Vandekerckhove, T. T., Claeys, M., Borgonie, G. & Gheysen, G. 2009 An endosymbiotic bacterium in a plant-parasitic nematode: member of a new *Wolbachia* supergroup. *Int. J. Parasitol.* **39**, 1045–1054. (doi:10.1016/j.ijpara.2009.01.006)

5 Werren, J. H., Baldo, L. & Clark, M. E. 2008 *Wolbachia*: master manipulators of invertebrate biology. *Nat. Rev. Microbiol.* **6**, 741–751. (doi:10.1038/nrmicro1969)

6 Hedges, L. M., Brownlie, J. C., O'Neill, S. L. & Johnson, K. N. 2008 *Wolbachia* and virus protection in insects. *Science* **322**, 702. (doi:10.1126/science.1162418)

7 Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X. Y. & Fukatsu, T. 2010 *Wolbachia* as a bacteriocyte-associated nutritional mutualist. *Proc. Natl Acad. Sci. USA* **107**, 769–774. (doi:10.1073/pnas.0911476107)

8 Teixeira, L., Ferreira, A. & Ashburner, M. 2008 The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biol.* **6**, e2. (doi:10.1371/journal.pbio.1000002)

9 Mathers, C. D., Ezzati, M. & Lopez, A. D. 2007 Measuring the burden of neglected tropical diseases: the global burden of disease framework. *PLoS Negl. Trop. Dis.* **1**, e114. (doi:10.1371/journal.pntd.0000114)

10 Hoerauf, A. *et al.* 1999 Tetracycline therapy targets intracellular bacteria in the filarial nematode *Litomosoides sigmodontis* and results in filarial infertility. *J. Clin. Invest.* **103**, 11–18. (doi:10.1172/JCI4768)

11 Langworthy, N. G., Renz, A., Mackenstedt, U., Henkle-Dührsen, K., de Bronsvoort, M. B., Tanya, V. N., Donnelly, M. J. & Trees, A. J. 2000 Macrofilaricidal activity of tetracycline against the filarial nematode *Onchocerca ochengi*: elimination of *Wolbachia* precedes worm death and suggests a dependent relationship. *Proc. R. Soc. Lond. B* **267**, 1063–1069. (doi:10.1098/rspb.2000.1110)

12 Hoerauf, A. 2008 Filariasis: new drugs and new opportunities for lymphatic filariasis and onchocerciasis. *Curr. Opin. Infect. Dis.* **21**, 673–681. (doi:10.1097/QCO.0b013e328315cde7)

13 Landmann, F., Foster, J. M., Slatko, B. & Sullivan, W. 2010 Asymmetric *Wolbachia* segregation during early *Brugia malayi* embryogenesis determines its distribution in adult host tissues. *PLoS Negl. Trop. Dis.* **4**, e758. (doi:10.1371/journal.pntd.0000758)

14 Keiser, P. B., Reynolds, S. M., Awadzi, K., Ottesen, E. A., Taylor, M. J. & Nutman, T. B. 2002 Bacterial endosymbionts of *Onchocerca volvulus* in the pathogenesis of posttreatment reactions. *J. Infect. Dis.* **185**, 805–811. (doi:10.1086/339344)

15 Foster, J. *et al.* 2005 The *Wolbachia* genome of *Brugia malayi*: endosymbiont evolution within a human pathogenic nematode. *PLoS Biol.* **3**, e121. (doi:10.1371/journal.pbio.0030121)

16 Fenn, K. & Blaxter, M. 2006 *Wolbachia* genomes: revealing the biology of parasitism and mutualism. *Trends Parasitol.* **22**, 60–65. (doi:10.1016/j.pt.2005.12.012)

17 Xie, H., Bain, O. & Williams, S. A. 1994 Molecular phylogenetic studies on filarial parasites based on 5S ribosomal spacer sequences. *Parasite* **1**, 141–151.

18 Nfon, C. K., Makepeace, B. L., Njongmeta, L. M., Tanya, V. N. & Trees, A. J. 2007 Lack of resistance after re-exposure of cattle cured of *Onchocerca ochengi* infection with oxytetracycline. *Am. J. Trop. Med. Hyg.* **76**, 67–72.

19 Gilbert, J., Nfon, C. K., Makepeace, B. L., Njongmeta, L. M., Hastings, I. M., Pfarr, K. M., Renz, A., Tanya, V. N. & Trees, A. J. 2005 Antibiotic chemotherapy of onchocerciasis: in a bovine model, killing of adult parasites requires a sustained depletion of endosymbiotic bacteria (*Wolbachia* species). *J. Infect. Dis.* **192**, 1483–1493. (doi:10.1086/462426)

20 Nfon, C. K., Makepeace, B. L., Njongmeta, L. M., Tanya, V. N., Bain, O. & Trees, A. J. 2006 Eosinophils contribute to killing of adult *Onchocerca ochengi* within onchocercomata following elimination of *Wolbachia*. *Microbes Infect.* **8**, 2698–2705. (doi:10.1016/j.micinf.2006.07.017)

21 Bazzocchi, C., Genchi, C., Paltrinieri, S., Lecchi, C., Mortarino, M. & Bandi, C. 2003 Immunological role of the endosymbionts of *Dirofilaria immitis*: the *Wolbachia* surface protein activates canine neutrophils with production of IL-8. *Vet. Parasitol.* **117**, 73–83. (doi:10.1016/j.vetpar.2003.07.013)

22 Brattig, N. W., Büttner, D. W. & Hoerauf, A. 2001 Neutrophil accumulation around *Onchocerca* worms and chemotaxis of neutrophils are dependent on *Wolbachia* endobacteria. *Microbes Infect.* **3**, 439–446. (doi:10.1016/S1286-4579(01)01399-5)

23 Tchakouté, V. L. *et al.* 2006 In a bovine model of onchocerciasis, protective immunity exists naturally, is absent in drug-cured hosts, and is induced by vaccination. *Proc. Natl Acad. Sci. USA* **103**, 5971–5976. (doi:10.1073/pnas.0601385103)

24 Striebel, H. P. 1988 Proposed form to evaluate some histological aspects of macrofilarial morphology, its age dependent alterations and drug related changes in nodules of *Onchocerca volvulus* and *O. gibsoni*. *Trop. Med. Parasitol.* **39**(Suppl. 4), 367–389.

25 Makepeace, B. L., Rodgers, L. & Trees, A. J. 2006 Rate of elimination of *Wolbachia pipientis* by doxycycline *in vitro* increases following drug withdrawal. *Antimicrob. Agents Chemother.* **50**, 922–927. (doi:10.1128/AAC.50.3.922-927.2006)

26 Hermans, P. G., Hart, C. A. & Trees, A. J. 2001 *In vitro* activity of antimicrobial agents against the endosymbiont

Wolbachia pipientis. *J. Antimicrob. Chemother.* 47, 659–663. (doi:10.1093/jac/47.5.659)

27 Townson, S., Tagboto, S., McGarry, H. F., Egerton, G. L. & Taylor, M. J. 2006 *Onchocerca* parasites and *Wolbachia* endosymbionts: evaluation of a spectrum of antibiotic types for activity against *Onchocerca gutturosa in vitro*. *Filaria J.* 5, 4. (doi:10.1186/1475-2883-5-4)

28 Kaiser, W., Huguet, E., Casas, J., Commin, C. & Giron, D. 2010 Plant green-island phenotype induced by leaf-miners is mediated by bacterial symbionts. *Proc. R. Soc. B* 277, 2311–2319. (doi:10.1098/rspb.2010.0214)

29 Barr, K. L., Hearne, L. B., Briesacher, S., Clark, T. L. & Davis, G. E. 2010 Microbial symbionts in insects influence down-regulation of defense genes in maize. *PLoS ONE* 5, e11339. (doi:10.1371/journal.pone.0011339)

30 Koppenhöfer, H. S. & Gaugler, R. 2009 Entomopathogenic nematode and bacteria mutualism. In *Defensive mutualism in microbial symbiosis* (eds J. F. White & M. S. Torres), pp. 99–116. Boca Raton, FL: CRC Press.

31 Brownlie, J. C., Cass, B. N., Riegler, M., Witsenburg, J. J., Iturbe-Ormaetxe, I., McGraw, E. A. & O'Neill, S. L. 2009 Evidence for metabolic provisioning by a common invertebrate endosymbiont, *Wolbachia pipientis*, during periods of nutritional stress. *PLoS Pathog.* 5, e1000368. (doi:10.1371/journal.ppat.1000368)

32 McNulty, S. N. *et al.* 2010 Endosymbiont DNA in endobacteria-free filarial nematodes indicates ancient horizontal genetic transfer. *PLoS ONE* 5, e11029. (doi:10.1371/journal.pone.0011029)

33 Plenge-Bönig, A., Krömer, M. & Büttner, D. W. 1995 Light and electron microscopy studies on *Onchocerca jakutensis* and *O. flexuosa* of red deer show different host-parasite interactions. *Parasitol. Res.* 81, 66–73. (doi:10.1007/BF00932419)

34 Wildenburg, G., Plenge-Bönig, A., Renz, A., Fischer, P. & Büttner, D. W. 1997 Distribution of mast cells and their correlation with inflammatory cells around *Onchocerca gutturosa*, *O. tarsicola*, *O. ochengi*, and *O. flexuosa*. *Parasitol. Res.* 83, 109–120. (doi:10.1007/s004360050220)